Pathogenic Potential of *Escherichia coli* O26 and Sorbitol-Fermenting *Escherichia coli* O157:NM

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Declaration

The research presented in this thesis is entirely my own work, except where otherwise stated. No part of this thesis has been submitted for a degree or professional qualification in any University.

Tracy Rosser March 2009

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List of abbreviations

α	alpha
A/E	attaching and effacing
aEPEC	atypical enteropathogenic *Escherichia coli*
APS	ammonium persulphate
~	approximately
β	beta
bp	base pairs
BSA	bovine serum albumin
CAM	chloramphenicol
CFA	colonisation factor antigen
CR	Congo red
dH_2O	distilled water
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EAEC	enteroaggregative *Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EHEC	enterohaemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
FBS	foetal bovine serum
γ	gamma
g	gram
GFP	green fluorescent protein
h	hour(s)
HRP	horseradish peroxidase
HUS	haemolytic uraemic syndrome
IL-	interleukin

kb	kilobase pairs
kDa	Kilodalton
LB	Luria-Bertani
LEE	locus of enterocyte effacement
M	Molar
MEM	minimal essential medium
mg	milligram (10^{-3} gram)
min	minute(s)
ml	millilitre (10^{-3} litre)
mM	millimolar (10^{-3} molar)
MLST	multilocus sequence typing
NEB	New England biolabs
NSF	non-sorbitol-fermenting
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
PFGE	pulsed-field gel electrophoresis
PRR	proline-rich repeat
RFU	relative fluorescence units
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SF	sorbitol-fermenting
ST	sequence type
T3SS	type III secretion system
TBE	Tris-borate/EDTA
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine

TFB	transformation buffer
TLR	toll-like receptor
tRNA	transfer ribonucleic acid
Tris	Trishydroxymethylaminomethane
µg	microgram (10^{-6} gram)
µl	microlitre (10^{-6} litre)
µM	micromolar (10^{-6} molar)
V	volt
VTEC	verocytotoxin-producing *Escherichia coli*
Vtx	verocytotoxin

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Abstract

Verocytotoxin-producing *Escherichia coli* (VTEC) are important human pathogens that may cause diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome (HUS). Worldwide, non-sorbitol-fermenting (NSF) VTEC O157:H7 is the most common serogroup associated with HUS but several non-O157:H7 serogroups have emerged as causes of this disease. This research investigated the pathogenic potential of two non-O157:H7 serogroups: O26 and sorbitol-fermenting (SF) O157:NM. While VTEC O26 have emerged as a significant cause of HUS in continental Europe, human infections associated with this pathogen are uncommon in Scotland and generally only result in simple diarrhoea. The study characterised *E. coli* O26 isolates recovered from human infections in Europe and Scotland and isolates collected from Scottish cattle with the objectives to identify factors which may allow strains to cause more serious clinical disease and to investigate the potential of bovine VTEC O26 in Scotland to cause human infection. MLST analysis of housekeeping genes found little genetic variation in the genomic 'backbone' among the vast majority of *E. coli* O26 isolates. The gene for verocytotoxin 2 (vtx_2) alone was carried by VTEC O26 isolates recovered from patients in continental Europe but was found in no Scottish human isolate, where the majority of isolates did not harbour a *vtx* gene. It was demonstrated that among the European VTEC O26 human isolates, 67% carried a specific allele within the promoter region for *LEE1* and 87% harboured the *tccP2* gene. In contrast, no Scottish VTEC O26 human isolate carried this allele or the *tccP2* gene. The impact these genotypic characteristics have on the pathogenic potential of a strain remains uncertain. There were no clear differences in verocytotoxin titres, levels of LEE-encoded protein secretion or levels of adherence to Caco-2 cells between VTEC O26 isolates recovered from human infections of varying severity. However, levels of LEE-encoded protein secretion from cattle isolates were generally higher than those from many of the human isolates. The differences in pathogenic potential between isolates are likely to be due to horizontally acquired DNA, including vtx_2 carriage and the O-island-phage-associated effector protein repertoire. Further work is required to determine if the differences identified may also impact on shedding levels

from cattle and therefore the likelihood of transmission to humans. Since 1988, SF VTEC O157:NM strains have emerged and have been associated with a higher incidence of progression to HUS than NSF VTEC O157:H7. This study investigated bacterial factors that may account for the increased pathogenic potential of SF VTEC O157:NM. While no evidence of toxin or toxin expression differences between the two VTEC O157 groups was found, the SF VTEC O157:NM strains adhered at significantly higher levels to a human colonic cell line. Under the conditions tested, curli were shown to be the main factor responsible for the increased adherence to Caco-2 cells. The capacity of SF VTEC O157:NM strains to express curli at 37^{o}C may have relevance to the epidemiology of human infections as curliated strains could promote higher levels of colonization and inflammation in the human intestine. In turn this could lead to increased toxin exposure and an increased likelihood of progression to HUS.

Chapter 1

Introduction

1.1 Bacterial evolution and pathogenesis

In examining the mechanisms that bacteria use to cause infections, it is worth pointing out that animals, including humans, and bacteria have evolved together. The majority of bacteria exist in a state of equilibrium with the host where commensal colonisation represents a beneficial circumstance for both. Additionally, animals may encounter pathogenic bacteria (that is disease-causing bacteria) many times a day, yet infection after such exposure is generally the exception rather than the rule. Of those who become infected some individuals will develop asymptomatic carriage and not all will progress to clinical disease. Bacterial disease is thus likely to occur in only a small proportion of the initially exposed population.

It is now accepted that many bacterial diseases are a consequence of a complex set of interactions between the bacteria and their host. Mammals have evolved many ways of protecting themselves from bacterial pathogens and the relatively high replication rate of most bacteria has meant they have evolved various mechanisms to overcome these innate and adaptive immune responses. Genome sequencing has revealed that strains within many bacterial species share a common genetic backbone which can be interspersed with 'islands' of DNA. This variable content is often associated with virulence capacity, either directly or by modulating an existing activity. Many of these islands show evidence of having been horizontally acquired from other bacteria via direct DNA uptake, conjugation or phage transduction, indicating many groups of bacteria have the capacity to share or exchange this information.

The Enterobacteriaceae family of Gram negative bacteria contain a number of genera of important pathogens that can produce infections in a wide range of hosts and elicit different types of pathologies. Even focusing down to one genus and one species such as *Escherichia coli* (*E. coli*) still provides a diverse group of bacteria that range from harmless commensals of the gastrointestinal tract to highly virulent pathogens. While the work described in this thesis is aimed at understanding differences in the pathogenic potential between related *E. coli* strains, it should be borne in mind that the mechanisms and evolutionary forces by which *E. coli* have evolved to survive in

its host either by causing disease, manipulating host defences or evading the immune response are relevant to many other pathogens, bacterial or otherwise.

1.2 Introduction to *E. coli*

E. coli is a Gram negative bacillus and is a normal constituent of the human colonic flora, typically colonising the infant gastrointestinal tract shortly after birth (Nataro & Kaper, 1998; Sussman, 1997). *E. coli* usually exist innocuously in the intestinal lumen and can be beneficial to the host. However, human disease can arise as: (i) normal, non-pathogenic strains, can cause infection in immunocompromised hosts and (ii) certain *E. coli* species are highly adapted and have evolved the ability to cause a wide spectrum of human diseases (Nataro & Kaper, 1998). *E. coli* is an important etiological agent in urinary tract infections, septicaemia, meningitis and diarrhoeal disease.

1.3 Diarrheagenic *E. coli*

E. coli strains associated with diarrhoeal disease can be categorised into five principal pathogenicity groups based on their virulence properties and mechanisms of pathogenicity (Wasteson, 2001):

(i) Enterotoxigenic *E. coli* (ETEC)

(ii) Enteroaggregative *E. coli* (EAEC)

(iii) Enteroinvasive *E. coli* (EIEC)

(iv) Enteropathogenic *E. coli* (EPEC)

(iv) Enterohaemorrhagic *E. coli* (EHEC)/Verocytotoxin-producing *E. coli* (VTEC)/Shiga toxin-producing *E. coli* (STEC)

1.3.1 Enterotoxigenic *E. coli* (ETEC)

ETEC strains colonise the surface of the small bowel mucosa and produce enterotoxins – namely, heat-labile toxins and heat-stable toxins – which result in watery diarrhoea. ETEC are associated with diarrhoea in travellers and infants in developing countries (Nataro & Kaper, 1998; Wasteson, 2001).

1.3.2 Enteroaggregative *E. coli* (EAEC)

EAEC are associated with persistent diarrhoea in developing populations and are defined by the fact that they have a characteristic adherence phenotype on HEp-2 cells, where the bacteria aggregate to each other in a stacked-brick configuration (Nataro & Kaper, 1998). While EAEC pathogenesis is not well understood, a three-stage model involving initial aggregative adherence followed by enhanced mucus production and then the production of an EAEC cytotoxin, which results in intestinal cell damage, has been suggested (Nataro & Kaper, 1998).

1.3.3 Enteroinvasive *E. coli* (EIEC)

EIEC produce disease in humans almost identical to the disease caused by *Shigella* spp. (Nataro & Kaper, 1998; Wasteson, 2001). Infection with EIEC results in watery diarrhoea and occasionally the patient experiences the dysentery syndrome, with blood, mucus and leukocytes in the stool, tenesmus and fever (Nataro & Kaper, 1998). EIEC invade and multiply within colonic epithelial cells (Wasteson, 2001) and have directional movement through the cytoplasm, extending into adjacent epithelial cells (Nataro & Kaper, 1998).

1.3.4 Enteropathogenic *E. coli* (EPEC)

EPEC is a major cause of infantile diarrhoea in developing countries (Nataro & Kaper, 1998; Wasteson, 2001). The central mechanism of EPEC pathogenesis is the attaching and effacing (A/E) lesion (Nataro & Kaper, 1998; Wasteson, 2001). This lesion is characterised by the effacement of microvilli, intimate adherence between the bacteria and the intestinal epithelium, accumulation of polymerised actin beneath the adherent bacteria and pedestal formation (Nataro & Kaper, 1998). Multiple mechanisms have been suggested for how diarrhoea results from infection with EPEC. The rapid onset of severe watery diarrhoea could be accounted for by the ability of EPEC to rapidly inactivate the sodium-$_D$-glucose cotransporter, which is responsible for the daily uptake of approximately 6 litres of fluid from the normal intestine (Dean *et al*., 2006). Additional mechanisms which may contribute to diarrhoea include disruption of barrier function and immune response-triggered secretory responses (Dean *et al*., 2006; Nataro & Kaper, 1998).

Typical EPEC are characterised by the presence of the EPEC adherence factor (EAF) plasmid (Trabulsi *et al.*, 2002). This plasmid carries the cluster of genes that encode bundle-forming pili (BFP), which interconnect bacteria within compact microcolonies and mediate localised adherence on cultured epithelial cells. EPEC lacking the EAF plasmid are referred to as atypical EPEC (aEPEC) (Trabulsi *et al.*, 2002).

1.3.5 Enterohaemorrhagic *E. coli* (EHEC)/Verocytotoxin-producing *E. coli* (VTEC)/Shiga toxin-producing *E. coli* (STEC)

1.3.5.1 Nomenclature

The term verocytotoxin-producing *E. coli* (VTEC) originates from the observation by Konowalchuk *et al.* (1977) that these strains produced a toxin which was cytotoxic to Vero cells. VTEC is synonymous with Shiga toxin-producing *E. coli* (STEC), a term which reflects the fact that one of the cytotoxins produced by these organisms is very similar in biological properties, physical properties and antigenicity to the Shiga toxin produced by *Shigella dysenteriae* (Nataro & Kaper, 1998). The term enterohaemorrhagic *E. coli* (EHEC) was originally devised to denote strains that cause haemorrhagic colitis and haemolytic uraemic syndrome (HUS), produce verocytotoxin, evoke A/E lesions on epithelial cells and possess a large plasmid (of ~ 60 MDa) (Nataro & Kaper, 1998; O'Brien & Kaper, 1998). Thus, EHEC represents a subset of VTEC and incorporates a clinical connotation that is not inferred with VTEC. Although not all VTEC are considered to be pathogenic, all EHEC strains are believed to be pathogens (Nataro & Kaper, 1998; Tarr *et al.*, 2005).

1.3.5.2 Human infection

Infection by VTEC can cause a spectrum of human diseases, ranging from asymptomatic carriage and mild diarrhoea to severe bloody diarrhoea and haemorrhagic colitis. Haemorrhagic colitis is characterised by severe abdominal cramps, watery diarrhoea followed by grossly bloody diarrhoea, and little or no fever (Nataro & Kaper, 1998; O'Brien & Kaper, 1998; Riley *et al.*, 1983). Human infection with VTEC strains is potentially fatal and may be associated with serious complications such as HUS (World Health Organisation, 1998). HUS is defined by a

triad of clinical features that include acute renal failure, thrombocytopaenia and microangiopathic haemolytic anaemia (Nataro & Kaper, 1998; O'Brien & Kaper, 1998). HUS is the principal cause of acute renal failure in children (O'Brien & Kaper, 1998; Tarr *et al.*, 2005). VTEC infections are most frequently observed in infants, children and elderly patients (World Health Organisation, 1998).

1.4 Typing methods for *E. coli*

1.4.1 Serotyping

The current scheme for the serological classification of *E. coli* is a modified version of that originally proposed by Kauffman in 1944. In accordance with the modified Kauffman scheme, *E. coli* are serotyped with respect to a number of antigens – namely, their somatic lipopolysaccharide O-antigen, flagellar H-antigen and capsular K-antigen (Nataro & Kaper, 1998; Sussman, 1997).

1.4.2 Biochemical typing

E. coli strains can be typed on the basis of their biochemical capabilities (Nataro & Kaper, 1998). Commercially available test strips (API 20E strips) are routinely used to characterise *E. coli* strains via the following biochemical reactions: production of β-galactosidase, arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulphide, urease and indole; utilisation of citrate; deamination of tryptophan; conversion of pyruvate to acetoin; hydrolysis of gelatin; and fermentation of glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and arabinose. However, the introduction of the Biolog Phenotype MicroArray system makes it possible to characterise strains with respect to a greater number of biochemical properties.

1.4.3 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is the current method of choice for the epidemiological subtyping of *E. coli* strains and is favoured because of its high level of discrimination (Noller *et al.*, 2003).

The digestion of genomic DNA with restriction enzymes that cut infrequently generates DNA fragments that are usually too large to separate by conventional agarose gel electrophoresis. However, these fragments can be effectively resolved by PFGE, which promotes the differential migration of large DNA fragments through agarose gels by periodically changing the direction of the electrical field (Sambrook *et al.*, 1989).

PFGE is designed to show maximal variation within a population. PFGE identifies the microvariation that is required to distinguish strains within a geographic area and is therefore useful for studying local epidemiology, such as investigations of localised outbreaks of disease (Maiden *et al.*, 1998). However, PFGE is too discriminatory for long term and global epidemiology (Maiden *et al.*, 1998).

1.4.4 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was first developed by Maiden *et al.* (1998) for the pathogen *Neisseria meningitidis* but has since been employed to study many other bacterial species (Cooper & Feil, 2004). MLST is a nucleotide sequence-based typing system that was established for analysing pathogenic bacteria from a global epidemiology perspective (Maiden *et al.*, 1998; Urwin & Maiden, 2003). MLST is based on the principles of multilocus enzyme electrophoresis (MLEE) (Selander *et al.*, 1986) but differs in that it characterises the alleles present at multiple house-keeping loci directly by nucleotide sequencing rather than indirectly via the electrophoretic mobility of the enzymes they encode (Maiden *et al.*, 1998). Like MLEE, MLST considers variation that accumulates slowly, and which is expected to be selectively neutral, and achieves high resolution by analysing multiple loci (Enright & Spratt, 1998; Maiden *et al.*, 1998). A major advantage of MLST over other molecular typing methods is that sequence data are unambiguous and can be accurately compared between different laboratories (Maiden *et al.*, 1998).

MLST uses the sequences of internal fragments of several (usually seven) housekeeping genes. Unique sequences for each gene are given arbitrary allele numbers as identifiers and, for each isolate, the combination of allele numbers at all

loci defines the allelic profile. Each unique allelic profile is assigned a sequence type (ST) number and related STs are assigned to the same ST complex (Maiden *et al.*, 1998; Wirth *et al.*, 2006).

1.5 *E. coli* serogroup O157

1.5.1 *E. coli* O157:H7

E. coli O157:H7 was first recognised as a human pathogen in 1982 following its association with two major disease outbreaks in the United States (Riley *et al.*, 1983). Since its identification as a pathogen, *E. coli* O157:H7 has been found to be the predominant VTEC serotype and the cause of a series of outbreaks in the United States, Canada, the United Kingdom and Japan (O'Brien & Kaper, 1998). Worldwide, the majority of the reported human illness outbreaks have been attributed to serotype O157:H7 (Bettelheim, 2003; Caprioli *et al.*, 1997; Tarr *et al.*, 2005) and this serotype has the strongest and most enduring aetiological association with HUS (Tarr *et al.*, 2005).

The recognition of *E. coli* O157:H7 as a pathogen has been facilitated by the availability of microbiological detection procedures based on the characteristic phenotypic feature of this organism, namely, its inability to ferment sorbitol (Mead & Griffin, 1998).

1.5.1.1 Sources of *E. coli* O157:H7 infection

Cattle and sheep are major reservoirs of EHEC, including *E. coli* O157:H7. These ruminants carry *E. coli* O157:H7 asymptomatically in their intestines and shed it in their faeces. Traditionally, many reported outbreaks of *E. coli* O157:H7 were food-borne and were associated with the consumption of foods, including beef and dairy products and salads (Centers for Disease Control and Prevention, 2000 & 2008; Tarr *et al.*, 2005; Upton & Coia, 1994; Willshaw *et al.*, 1994). These foods may have been contaminated directly with faeces or indirectly, through contaminated irrigation or via contamination during packaging and/or food preparation. Recent outbreaks have increasingly recognised environmental exposure as an important source of human infection. This includes contamination of water supplies from faeces of

animals (Licence *et al.*, 2001) and direct contact with farm animals and their faeces following visits to farms and recreational use of animal pasture (Strachan *et al.*, 2001). In addition, person to person transmission is also reported to be an infection route (Al-Jader *et al.*, 1999).

1.5.2 Sorbitol-fermenting (SF) *E. coli* O157:NM

In addition to *E. coli* O157:H7, strains of serotype O157:NM (nonmotile) which ferment sorbitol have emerged as important human pathogens in continental Europe (Karch & Bielaszewska, 2001).

1.5.2.1 SF VTEC O157:NM strains as human pathogens

SF VTEC O157:NM strains were first recognised during an outbreak of HUS in children in Bavaria, Germany, in 1988 (Karch & Bielaszewska, 2001). Since then, these strains have been isolated from patients throughout Germany (Gunzer *et al.*, 1992; Karch *et al.*, 1993), including a further outbreak in Bavaria that resulted in twenty-eight cases of HUS, three of them fatal (Ammon *et al.*, 1999). The first isolation of SF VTEC O157:NM strains outside Germany occurred in the Czech Republic in 1995 (Bielaszewska *et al.*, 1998). There have since been reports of this pathogen causing diarrhoea or HUS in Hungary (Karch & Bielaszewska, 2001), Finland (Keskimaki *et al.*, 1998), another region of the Czech Republic (Bielaszewska *et al.*, 2000), Austria (Orth *et al.*, 2006) and Sweden (S. Löfdahl, personal communication). Infection with SF VTEC O157:NM was not reported outside continental Europe until 2002 when it was isolated in Australia (Bettelheim *et al.*, 2002) and Scotland (Allison, 2002). It was subsequently isolated from patients in England (HPA, 2006) and Ireland (Garvey *et al.*, 2006).

1.5.2.2 Sources of SF VTEC O157:NM infection

The reservoirs and transmission routes of SF VTEC O157:NM remain largely unknown. While cattle are well established as a major reservoir of VTEC O157:H7, there are only two reports of the isolation of SF VTEC O157:NM strains from cattle (Bielaszewska *et al.*, 2000; Orth *et al.*, 2006). Although more than 1,300 bovine faecal samples have been examined for SF VTEC O157:NM in Germany and the

Czech Republic (Karch & Bielaszewska, 2001), only one was found to be positive for this pathogen (Bielaszewska *et al.*, 2000). In addition, except for a single isolation of a SF VTEC O157:NM strain from a pony, this pathogen has not been isolated from any other domestic or wild animal, including sheep, goats and deer, in continental Europe (Karch & Bielaszewska, 2001). However, SF VTEC O157:NM has been isolated from cat faeces in Scotland (Locking *et al.*, 2004). Since SF VTEC O157:NM have only rarely been isolated from animals, it has been suggested that these pathogens might be adapted to the human intestine and that humans may be their principal reservoir (Karch & Bielaszewska, 2001).

The routes of transmission of SF VTEC O157:NM infection are unknown in most cases. However, the main transmission routes of VTEC O157:H7 infection have also been identified for SF VTEC O157:NM. A large outbreak of HUS caused by SF VTEC O157:NM in Germany probably had a food-borne origin (Ammon *et al.*, 1999). Case-control studies identified two sausages, namely mortadella and teewurst, the latter of which contains raw beef, as likely sources of infection (Ammon *et al.*, 1999). In addition, a report from Austria described the isolation of SF VTEC O157:NM from a child with HUS who had consumed unpasteurised cow's milk (Allerberger *et al.*, 2001). Direct contact with animals, including a cow (Bielaszewska *et al.*, 2000) and a pony (Karch & Bielaszewska, 2001) that shed SF VTEC O157:NM in their faeces, was the most probable route of transmission of infection to patients in two instances (Bielaszewska *et al.*, 2000; Karch & Bielaszewska, 2001). Moreover, SF VTEC O157:NM was isolated from cat faeces at a farm where a bloody diarrhoeal infection had occurred and the cat isolate shared an indistinguishable PFGE profile with the clinical isolate (Locking *et al.*, 2004). Finally, person to person transmission was suggested to be the route of SF VTEC O157:NM infection of a 10 month old child in Austria (Orth *et al.*, 2006).

1.5.3 Evolutionary model of *E. coli* O157

In the evolutionary model proposed by Feng *et al.* (1998; 2007), both VTEC O157:H7 and SF VTEC O157:NM evolved from a common EPEC-like O55:H7 ancestor that was able to ferment sorbitol (SOR^+) and express β-glucuronidase

(GUD$^+$) and that carried the LEE (locus of enterocyte effacement) pathogenicity island. This *E. coli* O55:H7 ancestor is proposed to have acquired the gene for verocytotoxin 2 (*vtx*$_2$), a large plasmid and the *rfb* region encoding O157 antigen, resulting in a hypothetical intermediate O157:H7 Vtx2-producing strain that retained the SOR$^+$ GUD$^+$ phenotype (Feng *et al.*, 1998). From this *E. coli* O157:H7 ancestor, the model proposes that two distinct lineages evolved. In one path, the loss of the ability to ferment sorbitol and express β-glucuronidase and the acquisition of the *vtx*$_1$ gene led to the emergence of the prototypic non-sorbitol-fermenting (NSF) VTEC O157:H7 clone (Feng *et al.*, 1998). Along the other path, the loss of motility yielded the SF VTEC O157:NM clone, which retained the ancestral ability to ferment sorbitol, express β-glucuronidase and produce Vtx2 (Feng *et al.*, 1998).

1.6 Non-O157 VTEC

1.6.1 Non-O157 VTEC in human disease

More than 200 non-O157 VTEC serotypes have been reported and over 100 of them have been associated with human illness (World Health Organisation, 1998). It has only lately been appreciated that non-O157 VTEC are important pathogens, capable of causing a spectrum of disease in humans comparable to that produced by serotype O157:H7 (World Health Organisation, 1998). However, the prevalence of infections with non-O157 VTEC strains is probably underestimated due to the failure of routinely used diagnostic procedures, such as sorbitol MacConkey agar, to detect non-O157 VTEC serotypes (Bettelheim, 2003; Karch *et al.*, 1999; World Health Organisation, 1998).

In the United States, Canada, the United Kingdom and Japan, the prevalence of non-O157 VTEC is low, much lower than that of VTEC O157:H7. However, in parts of the Southern hemisphere and continental Europe, non-O157 VTEC are significant causes of human disease, being responsible for a series of outbreaks and sporadic cases (Blanco *et al.*, 2004; Caprioli *et al.*, 1994; Caprioli *et al.*, 1997; Elliott *et al.*, 2001; Paton *et al.*, 1996; Robins-Browne *et al.*, 1998; Tozzi *et al.*, 2003; Werber *et al.*, 2002).

Among European countries, the proportions of VTEC infections due to VTEC O157 and VTEC non-O157 reported through Enter-net (an international surveillance network for human gastrointestinal infections) for 2005 are shown in Table 1.1. These data show considerable differences in the ratio between cases of VTEC O157 and non-O157 VTEC infections in different countries. For example, the vast majority of infections detected in Scotland, England and Wales were attributed to VTEC O157. In contrast, in continental Europe, many of the infections were due to non-O157 serotypes, although there were marked national differences. VTEC O157 is the most prevalent serotype detected in Belgium, France, the Netherlands and Spain whereas in Denmark, Germany, Italy, Luxembourg and Norway other serotypes are more commonly detected. While some of these differences are likely to reflect the diagnostic and surveillance strategies in these countries, they may also reflect natural differences in the occurrence of these pathogens.

Table 1.1. Numbers of reported VTEC cases, proportions due to VTEC O157 and VTEC non-O157 and numbers of cases of HUS for 2005 (Enter-net, 2005)[a].

Country	Number of cases	% of cases due to		Number of HUS cases[b]
		O157	Non-O157	
Belgium	52	60%	40%	20
Denmark	156	17%	72%[c]	4
England and Wales	954	100%		NK[d]
France	108	68%	19%[c]	20
Germany	759	10%	64%[c]	8
Ireland	125	86%	14%	17
Italy	22	14%	82%[c]	19
Luxemburg	11	18%	82%	NK[d]
Netherlands	54	100%		4
Norway	18	39%	50%[c]	1
Scotland	176	94%	6%	NK[d]
Spain	15	100%		1

[a] Table adapted from Panel on Biological Hazards (2007).
[b] HUS is a clinical diagnosis, hence cases are also reported in the absence of the isolation or confirmation of VTEC infection.
[c] Isolates from some cases not serotyped.
[d] Not known.

Among the numerous non-O157 VTEC serotypes, strains belonging to serogroups O26, O103, O111 and O145 are more commonly associated with serious disease, such as haemorrhagic colitis and HUS, and are thus clearly recognised as human pathogens (Brooks *et al.*, 2005; Nataro & Kaper, 1998; World Health Organisation, 1998).

1.6.2 Prevalence of non-O157 VTEC in cattle

A recent study investigated the prevalence of *E. coli* serogroups O26, O103, O111 and O145 in faeces of cattle in Scotland (Pearce *et al.*, 2006). It was reported that no *E. coli* O111 were detected and, while *E. coli* O103 and O145 were isolated from the faeces of Scottish cattle, the carriage of verocytotoxin genes in these strains was rare (Pearce *et al.*, 2006). In contrast, VTEC O26 strains were common and widely dispersed in Scottish cattle (Pearce *et al.*, 2006).

1.6.3 *E. coli* serogroup O26

E. coli serogroup O26 comprises two major groups of strains. One group consists of strains that possess the verocytotoxin genes and are classified as VTEC. The other group comprises *E. coli* O26 strains that lack the verocytotoxin genes and are classified as aEPEC as they have the ability to form A/E lesions on the intestinal epithelium but lack the EAF plasmid (Nataro & Kaper, 1998).

Although the prevalence of VTEC O26 remains low in relation to VTEC O157:H7 in the United Kingdom, VTEC O26 has emerged as an important cause of human disease in other countries (Caprioli *et al.*, 1997; Tozzi *et al.*, 2003; Werber *et al.*, 2002; Zhang *et al.*, 2000).

1.6.3.1 Sources of *E. coli* O26 infection

The source of VTEC O26 infections are rarely found (Jenkins *et al.*, 2008; Zhang *et al.*, 2000). However, there is evidence to suggest that food-borne transmission was the most likely route for a number of VTEC O26 infections (Allerberger *et al.*, 2003; Werber *et al.*, 2002). In addition, since VTEC O26 have been isolated from the faeces of cattle, pigs, sheep, goats and chickens (Jenkins *et al.*, 2008; Leomil *et al.*,

2005; Pearce *et al.*, 2006), their transmission to humans directly or indirectly from animals and their faeces is also a possible route of infection (Jenkins *et al.*, 2008).

1.6.3.2 *E. coli* O26 in animals

E. coli O26 are also of veterinary importance. While they have been isolated from a variety of healthy animals, including cattle and pigs (Jenkins *et al.*, 2008; Pearce *et al.*, 2004, 2006), they are commonly associated with diarrhoea in calves (Pearson *et al.*, 1999; Wieler *et al.*, 1996). *E. coli* O26 are often recovered from diarrhoeic calves which are simultaneously infected with *Cryptosporidium* or are otherwise immunocompromised (R. La Ragione, personal communication).

1.7 VTEC virulence factors

1.7.1 Verocytotoxins

The ability to produce verocytotoxin (Vtx) is a major virulence trait of all VTEC. The structural genes for Vtx are encoded on lysogenic bacteriophages which have inserted into the chromosome. Two major toxin types, Vtx1 and Vtx2, have been described. The structural genes of Vtx1 and Vtx2 only share 56% amino acid sequence homology and the two toxins are antigenically distinct (Nataro & Kaper, 1998). A single VTEC isolate may produce Vtx1 alone, Vtx2 alone or both of these toxins in combination (Melton-Celsa & O'Brien, 1998).

1.7.1.1 Structure and mode of action

Both Vtx1 and Vtx2 have a conserved A_1B_5 subunit structure, in which one A subunit is noncovalently associated with a pentamer of B subunits (O'Brien & Holmes, 1987). The ~32 kDa A subunit is proteolytically cleaved to yield a ~28 kDa peptide (A_1) and a ~4 kDa peptide (A_2), which remain linked by a disulphide bond. The A_1 peptide contains the enzymatic activity, while the A_2 peptide binds the A subunit to a pentamer of identical ~7.7 kDa B subunits (Nataro & Kaper, 1998). The B pentamer binds the toxin to a specific glycolipid receptor, typically globotriaosylceramide (Gb_3), present on the surface of eukaryotic cells. The toxin is subsequently endocytosed through coated pits and transported to the Golgi apparatus and then to the endoplasmic reticulum (Nataro & Kaper, 1998). The A subunit is

translocated to the cytoplasm where it acts on the 60S ribosomal subunit – the A_1 peptide is an *N*-glycosidase that removes a single adenine residue from the 28S rRNA of eukaryotic ribosomes, thereby preventing protein synthesis (Nataro & Kaper, 1998). The disruption of protein synthesis causes cell death (Melton-Celsa & O'Brien, 1998; Nataro & Kaper, 1998).

1.7.1.2 Vtx1

Vtx1 originally appeared to be highly conserved. The nucleotide sequences for vtx_1 from three different bacteriophages, H19B (De Grandis *et al.*, 1987), H30 (Kozlov Yu *et al.*, 1988) and 933J (Jackson *et al.*, 1987), are identical and are very similar to the nucleotide sequence of *stx* from *Shigella dysenteriae* type 1 (Strockbine *et al.*, 1988). However, several variants of Vtx1 have since been described. Paton *et al*. (1993, 1995) identified three minor vtx_1 variants which shared more than 99% nucleotide sequence identity with vtx_1 from phage 933J and encoded Vtx1 proteins which differed from Vtx1 encoded by phage 933J by only two amino acid residues in their A subunits. In contrast, the vtx_1 variants, vtx_{1c} and vtx_{1d}, differ considerably from the classical vtx_1. vtx_{1c}, present in VTEC strains of human and ovine origin, differs from vtx_1 of phage 933J by 43 nucleotides, resulting in changes of nine amino acids in the A subunit and three amino acids in the B subunit (Paton *et al.*, 1995; Zhang *et al.*, 2002). vtx_{1d}, identified in a VTEC strain isolated from cattle, has only 91% nucleotide sequence homology with vtx_1 from phage 933J, resulting in changes of twenty and seven amino acid residues in the A and B subunits, respectively (Burk *et al.*, 2003).

1.7.1.3 Vtx2

The Vtx2 group is highly heterogeneous, containing Vtx2 and several Vtx2 variants including Vtx2c (Schmitt *et al.*, 1991), Vtx2d (Pierard *et al.*, 1998), Vtx2e (Weinstein *et al.*, 1988), Vtx2f (Schmidt *et al.*, 2000) and Vtx2g (Leung *et al.*, 2003). While Vtx2, Vtx2c, Vtx2d and Vtx2e can all be produced by VTEC strains isolated from humans (Friedrich *et al.*, 2002; Pierard *et al.*, 1998; Schmitt *et al.*, 1991), VTEC strains possessing different vtx_2 variants appear to differ in their pathogenic potential (Friedrich *et al.*, 2002). VTEC containing vtx_2 or vtx_{2c} are associated with

HUS whereas strains possessing *vtx*$_{2d}$ or *vtx*$_{2e}$ are less virulent (Friedrich *et al.*, 2002). VTEC isolates harbouring *vtx*$_2$ variants *vtx*$_{2d}$, *vtx*$_{2e}$ and *vtx*$_{2f}$ are typically recovered from sheep (Pierard *et al.*, 1998; Ramachandran *et al.*, 2001), pigs (Weinstein *et al.*, 1988) and pigeons (Schmidt *et al.*, 2000), respectively.

1.7.1.4 Role of Vtx in human disease

Vtx is thought to be the principal factor responsible for the intestinal manifestations (bloody diarrhoea and haemorrhagic colitis) and systemic complications (HUS) following VTEC infection (World Health Organisation, 1998). There is evidence that VTEC producing Vtx2 are more likely to cause HUS than VTEC producing only Vtx1 (Boerlin *et al.*, 1999; Brooks *et al.*, 2005; Ethelberg *et al.*, 2004; Jenkins *et al.*, 2003; Ostroff *et al.*, 1989; Scotland *et al.*, 1987). These observations have been supported in both a mouse model (Tesh *et al.*, 1993) and primate animal model (Siegler *et al.*, 2003). Tesh *et al*. (1993) found that Vtx2 was more toxic to mice than Vtx1 and Siegler *et al.* (2003) reported that the administration of purified Vtx2, but not of Vtx1, caused HUS in experimentally treated baboons.

A number of observations indicate that the amount of Vtx produced by infecting VTEC strains may correlate with the clinical outcome of infection (Eklund *et al.*, 2002; Muniesa *et al.*, 2003). However, others did not see this phenomenon (Cornick *et al.*, 2002; Zhang *et al.*, 2005).

1.7.1.4.1 Vtx in intestinal disease

It has been suggested that diarrhoea results from the local action of Vtx on the intestinal mucosa, with Vtx selectively killing the absorptive villus tip intestinal epithelial cells (Karmali, 1989; Nataro & Kaper, 1998). In rabbit ileum, the Gb_3 receptor is present in much higher concentrations in villus cells than in secretory crypt cells and so the death of absorptive cells and proliferation of secretory crypt cells could result in intestinal secretion instead of intestinal absorption which normally occurs (Nataro & Kaper, 1998). However, it has also been proposed that bloody diarrhoea, and possibly even diarrhoea, is caused by mesenteric ischaemia

initiated by circulating toxin, rather than by direct VTEC injury of the intestinal epithelium (Tarr *et al.*, 2005).

1.7.1.4.2 Vtx in HUS

The most important target of Vtx in the development of HUS is the renal microvascular endothelium. However, how Vtx is transported from the intestine to its extraintestinal target organ is uncertain. It has been suggested that Vtx might be transported on the surface of circulating neutrophils (Brigotti *et al.*, 2006; Griener *et al.*, 2007; te Loo *et al.*, 2000). However, others have been unable to reproduce this finding (Flagler *et al.*, 2007; Geelen *et al.*, 2007).

Following transport to its target organ, Vtx binds to Gb_3 receptors, which occur on renal glomerular endothelial, mesangial and tubular epithelial cells (Tarr *et al.*, 2005). Vtx is believed to damage the glomerular endothelial cells, and this injury generates thrombin, and fibrin is deposited in the microvasculature. The resultant decrease in the rate of glomerular filtration is assumed to be responsible for the acute renal failure which is typical of HUS (Nataro & Kaper, 1998).

There are many potential outcomes of HUS, the most severe being death. However, fortunately, most patients do recover from HUS, but there are concerns about their long-term renal function and it is possible that some patients will require dialysis (Tarr *et al.*, 2005).

1.7.2 Attaching and effacing (A/E) lesion formation

Central to the pathogenesis of VTEC is their ability to colonise the intestinal mucosa via the formation of A/E lesions. The A/E lesion is characterised by effacement of epithelial cell microvilli, intimate adherence of the bacterium to the epithelial cell membrane and the formation of an actin-rich pedestal-like structure in the host cell underneath the membrane at the site of attachment (Kaper *et al.*, 1998; Nataro & Kaper, 1998) (Figure 1.1). The genes required for the formation of A/E lesions are carried on a 36 kb pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995).

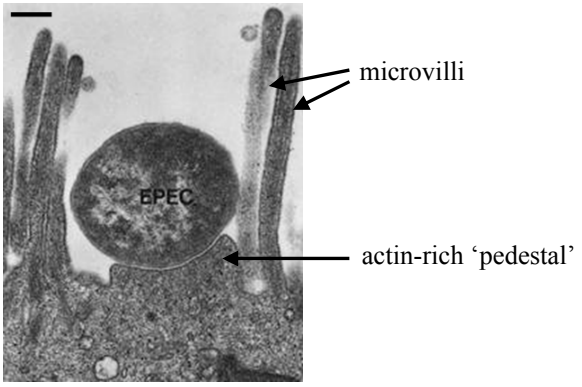


Figure 1.1. Electron micrograph showing a characteristic attaching and effacing (A/E) lesion. There is intimate attachment of the bacterium to the epithelial cell membrane following effacement of epithelial cell microvilli. The bacterium appears to be sitting on a pedestal-like structure on the host cell. Magnification bar, 0.4 μm. Image reproduced from Knutton *et al.* (1987).

1.7.2.1 Locus of enterocyte effacement (LEE)

The LEE has a considerably lower G and C content in comparison to the average *E. coli* genome, indicating that the LEE was likely acquired from another species by horizontal gene transfer (Frankel *et al.*, 1998). Three tRNA genes (*selC*, *pheU* and *pheV*) are known as integration sites of the LEE (Jores *et al.*, 2004). In EPEC O127:H6 strain E2348/69 and EHEC O157:H7 the LEE is inserted into the chromosome at the *selC* tRNA locus (McDaniel *et al.*, 1995; Wieler *et al.*, 1997) and in *E. coli* O26 strains the LEE is inserted into the *pheU* gene (Jores *et al.*, 2004). The LEE is not only necessary but also sufficient for the formation of A/E lesions by EPEC. McDaniel and Kaper (1997) reported that cloning the entire LEE of EPEC E2348/69 into *E. coli* K-12 was sufficient to enable them to form A/E lesions. In contrast, the LEE of EHEC O157:H7 failed to produce A/E lesions when cloned into the same K-12 strain (Elliott *et al.*, 1999). It has since been demonstrated that non-LEE encoded TccP is also required for actin polymerisation by EHEC O157:H7 (Campellone *et al.*, 2004; Garmendia *et al.*, 2004) (discussed in more detail in section 1.7.2.2.1).

The LEE contains 41 open reading frames (ORFs) organised in five operons, *LEE1* to *LEE5* (Figure 1.2). The LEE carries the genes encoding intimin, a type III

secretion system (T3SS), a number of secreted (Esp) proteins and the translocated intimin receptor, Tir (Nataro & Kaper, 1998). The outer membrane protein intimin, encoded by the *eae* gene, is responsible for the intimate adherence of bacteria to epithelial cells. The secreted proteins, EspA, EspB and EspD, are involved in the formation of a translocon, through which effector proteins can transfer into the host cell (Frankel *et al.*, 1998). Tir, which is one of the translocated proteins, is inserted in the host cell membrane, where it serves as a receptor to intimin.

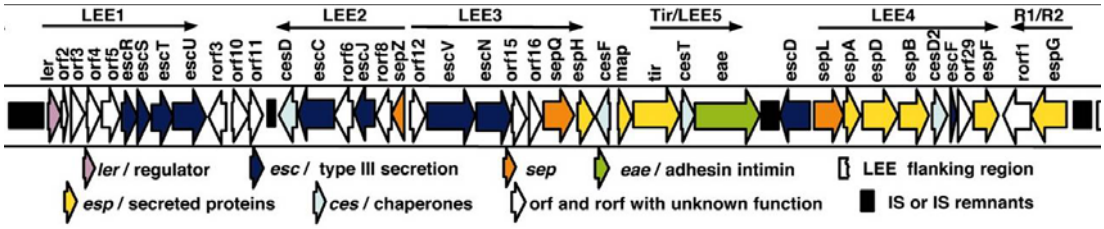


Figure 1.2. The genetic organisation of the locus of enterocyte effacement (LEE). The diagram details the LEE of *Citrobacter rodentium*, which is very similar to that of EHEC O157:H7 and EPEC. Image reproduced from Deng *et al.* (2004).

1.7.2.2 Model of A/E lesion formation

Initial contact between the bacterium and the epithelial cell stimulates the expression of LEE-encoded proteins and the formation of a translocon apparatus. The translocon consists of a hollow EspA filament which connects an EscC-generated pore in the bacterial envelope and an EspB/EspD-generated pore in the host cell membrane, thus providing a continuous but gated channel from the bacterial cytoplasm to the host cell (Figure 1.3). Tir is translocated into the host cell, where it becomes inserted into the plasma membrane in a hairpin loop topology (Hartland *et al.*, 1999). The extracellular Tir loop serves as a receptor for the bacterial outer membrane protein intimin [reviewed in Frankel *et al*. (2001)] while the intracellular amino and carboxy termini interact with several focal adhesion and cytoskeletal proteins, linking the extracellular bacterium to the host-cell cytoskeleton (Campellone & Leong, 2003; Goosney *et al.*, 2001). The clustering of Tir in the host

cell membrane following intimin binding triggers a signalling cascade leading to the formation of actin-rich pedestals underneath adherent bacteria.

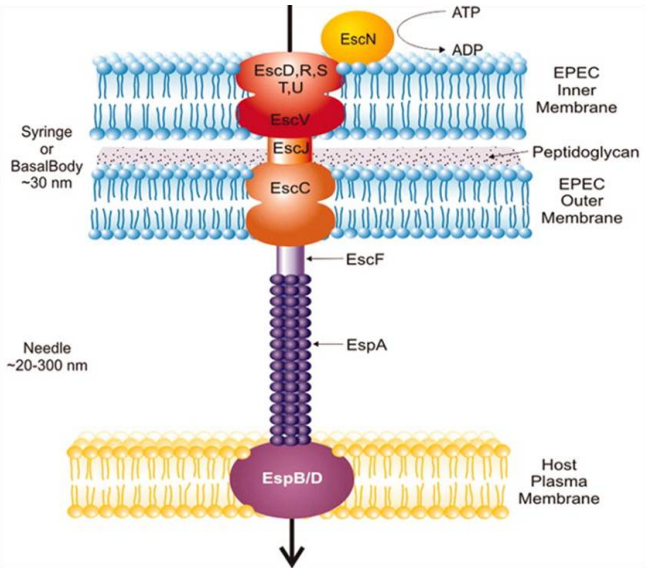


Figure 1.3. Translocon apparatus of the type III secretion system. The translocon consists of a hollow EspA filament which connects an EscC-generated pore in the bacterial outer membrane and an EspB/EspD-generated pore in the host cell membrane, thus providing a continuous but gated channel from the bacterial cytoplasm to the host cell. Image reproduced from Gauthier *et al*. (2003).

1.7.2.2.1 Actin polymerisation by EHEC and EPEC

Until recently, it was considered that localised actin polymerisation by EHEC and EPEC is achieved by distinct signal-transduction pathways. Actin assembly by EPEC O127:H6 strain E2348/69 requires phosphorylation of tyrosine Y474 of the intracellular carboxy-terminal domain of Tir (Kenny, 1999), which is present in the context of the consensus binding site (YpDEP/D/V) for the mammalian adaptor protein Nck. Tyrosine phosphorylated Tir recruits Nck (Campellone *et al.*, 2002; Gruenheid *et al.*, 2001), which in turn binds and activates the neuronal Wiskott-Aldrich syndrome protein (N-WASP). N-WASP activates the actin-related protein 2/3 (Arp2/3) complex, triggering actin polymerisation and pedestal formation [reviewed in Garmendia *et al.* (2005a)]. In addition, Tir EPEC O127:H6 can also initiate actin polymerisation by an Nck-independent mechanism through

phosphorylation of a second carboxy-terminal tyrosine residue, Y454 (Campellone & Leong, 2005). This pathway generates actin pedestals at a much reduced efficiency compared with the Nck pathway and the putative adaptor for the recruitment of N-WASP and Arp2/3 in this minor pathway is currently unknown.

In contrast to the above, Tir EHEC O157:H7 lacks a Y474 equivalent and is not tyrosine phosphorylated. Instead of Nck, EHEC O157:H7 utilises a second bacterial T3SS effector protein, TccP (Tir-cytoskeleton coupling protein) (also termed $EspF_U$) (Campellone *et al.*, 2004; Garmendia *et al.*, 2004). TccP mimics Nck in terms of recruitment and activation of N-WASP to trigger localised actin polymerisation. A 12-residue peptide within the carboxy-terminal of Tir EHEC O157:H7 has been shown to be necessary and sufficient to recruit TccP and initiate actin polymerisation (Campellone *et al.*, 2006). This peptide includes Y458, which is the equivalent of Y454 in Tir EPEC O127:H6 that has been implicated in the inefficient Nck-independent actin polymerisation pathway (Brady *et al.*, 2007; Campellone *et al.*, 2006). Importantly, TccP does not appear to bind Tir directly (Campellone *et al.*, 2004; Garmendia *et al.*, 2004), but the identity of the putative linker protein remains unknown. TccP is only the second effector protein after Tir that plays a direct role in actin polymerisation by EHEC O157:H7 (Frankel & Phillips, 2008).

It has become apparent that the distinction between the EPEC and EHEC actin polymerisation pathways is not absolute. Analysis of a large number of clinical and environmental isolates revealed that *tccP* is not confined to EHEC O157:H7; *tccP* is also present in some non-O157 EHEC and EPEC strains (Garmendia *et al.*, 2005b). Further investigation of a representative *tccP*-positive EPEC O119:H6 strain (ICC199) revealed that Tir is tyrosine (Y473) phosphorylated and can simultaneously utilise both the Nck and TccP pathways (Whale *et al.*, 2006).

1.7.2.2.2 TccP

The gene *tccP* (Z3072/ECs2715) is carried on prophage CP-933U/Sp14, forming an operon with the upstream gene *espJ* (Garmendia & Frankel, 2005), which encodes another effector protein. TccP consists of a unique 80 amino acid amino-terminal

region, that is involved in protein translocation (Garmendia *et al.*, 2006), and several almost identical 47 amino acid proline-rich repeats (PRRs) (Campellone *et al.*, 2004; Garmendia *et al.*, 2006). The two sequenced EHEC O157:H7 strains, EDL933 and Sakai (Hayashi *et al.*, 2001; Perna *et al.*, 2001), contain a *tccP* gene that encodes a unique amino-terminus and five PRRs and the beginning of a sixth repeat. Examination of numerous *E. coli* O157:H7 isolates revealed that the number of PRRs can vary between two and five (Garmendia *et al.*, 2005b). It has been shown that a TccP variant containing the unique amino-terminus and the first two PRRs is the smallest derivative of TccP capable of triggering host cell actin polymerisation (Garmendia *et al.*, 2006).

1.7.2.2.3 TccP2

In addition to *tccP*, the sequenced EHEC O157:H7 strains also contain a pseudogene, *tccP2* (*Z1385/ECs1126*), which is carried on prophage CP-933M/Sp4. A single base pair deletion at position 28 introduces a translation frame shift and a premature stop codon. Indeed, deletion of *tccP2* in EHEC O157:H7 EDL933 has no effect on actin polymerisation activity (Garmendia *et al.*, 2004). It has recently been found that SF EHEC O157:NM and the majority of non-O157 EHEC (serogroups O26, O103, O111, O121 and O145) harbour an intact functional *tccP2* gene (Ogura *et al.*, 2007). A study of the prevalence of *tccP2* in typical EPEC isolates found that while all EPEC lineage 1 strains (characterised by the expression of flagellar antigen H6 and intimin α) analysed were *tccP2* negative, all but one of the EPEC lineage 2 strains (characterised by the expression of flagellar antigen H2 and intimin β) tested contained intact *tccP2* (Whale *et al.*, 2007).

TccP2 consists of a unique 80 amino acid amino terminus, which shares 42.7% sequence identity and 69.5% sequence similarity with the amino terminus of TccP (Frankel & Phillips, 2008), and several PRRs that are almost identical to those of TccP (Ogura *et al.*, 2007). Similarly to TccP, variation in the number of TccP2 PRRs between strains has been noted (Ogura *et al.*, 2007). Functional studies have revealed that TccP2 is translocated by the LEE-encoded T3SS, and that TccP2 is

functionally equivalent to TccP as TccP2 restored actin polymerisation ability to EDL933Δ*tccP* (Ogura *et al.*, 2007; Whale *et al.*, 2007).

1.7.3 Fimbriae

Adherence is a necessary prerequisite for bacterial colonisation and pathogenesis. Fimbrial adhesins are important virulence factors that mediate adherence of the bacteria to cells of the gastrointestinal tract. At least 16 putative fimbrial operons have been identified in *E. coli* O157:H7 (Hayashi *et al.*, 2001; Low *et al.*, 2006; Perna *et al.*, 2001), including the well-characterised type 1 fimbriae and curli adhesins.

1.7.3.1 Type 1 fimbriae

Type 1 fimbriae are the most common adhesin produced by *E. coli* (Leathart & Gally, 1998) (Figure 1.4) and mediate adherence to mannose-containing glycoproteins found on the surfaces of many eukaryotic cells. Type 1 fimbriae are a proven virulence factor for uropathogenic *E. coli*, playing an important role in their colonisation of the human urinary tract (Holden *et al.*, 2006).

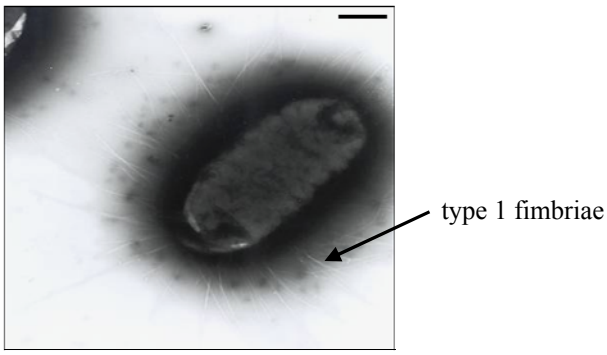


Figure 1.4. Electron micrograph of a bacterium expressing type 1 fimbriae. Magnification bar, 0.4 μm. Image courtesy of Prof D. Gally.

The expression of type 1 fimbriae is phase variable and depends on an invertible DNA element (*fim* switch) (Abraham *et al.*, 1985) that contains the promoter for *fimA*, the gene which encodes the main fimbrial subunit. When the *fim* switch is in the off orientation, type 1 fimbriae cannot be produced. Inversion of the *fim* switch

requires two recombinases, FimB and FimE, as well as numerous co-factors (Gally *et al.*, 1996). *E. coli* O157:H7 are unable to produce type 1 fimbriae because they contain a 16 bp deletion in the *fim* switch (Li *et al.*, 1997; Roe *et al.*, 2001), which prevents inversion of the *fim* switch to the on orientation.

1.7.3.2 Curli

Many strains of *E. coli* express thin aggregative fimbriae called curli (Barnhart & Chapman, 2006) (Figure 1.5). Curli expression is optimal at temperatures below 30°C and under conditions of low nutrients, low osmolarity and stationary growth (Barnhart & Chapman, 2006).

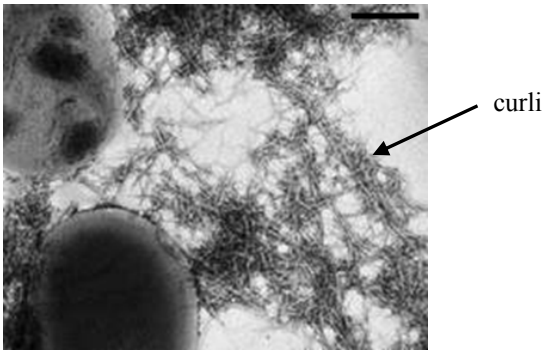


Figure 1.5. Electron micrograph of bacteria expressing curli. Magnification bar, 0.4 μm. Image reproduced from Barnhart & Chapman (2006).

There are several lines of evidence which suggest that curli are important during human infection. Firstly, curli bind to the extracellular matrix proteins fibronectin and laminin (Barnhart & Chapman, 2006; Olsen *et al.*, 1989) and have been shown to contribute to the attachment and invasion of host cells (Kikuchi *et al.*, 2005; Kim & Kim, 2004; Uhlich *et al.*, 2002). Thus, curli are likely to play an important role in the initial stages of the infection process (Barnhart & Chapman, 2006). Secondly, curli interact with numerous host proteins, many of which may facilitate the spread of the bacteria through the host (Barnhart & Chapman, 2006). Curliated bacteria bind plasminogen and tissue type plasminogen activator simultaneously, which results in the activation of plasminogen to plasmin, a serine protease that degrades soft tissue (Barnhart & Chapman, 2006; Sjobring *et al.*, 1994). In addition, curli can

bind to human contact-phase proteins, including fibrinogen, and thus slow clotting (Barnhart & Chapman, 2006). Thirdly, curli can activate the innate immune system. Curli have been shown to be a pathogen-associated molecular pattern (PAMP) that is recognized by Toll-like receptor 2 (TLR2) and results in the activation of interleukin-8 (IL-8) (Tukel *et al.*, 2005). This is consistent with an earlier study which demonstrated that curli induce the proinflammatory cytokines tumour necrosis factor alpha (TNF-α), IL-6 and IL-8 (Bian *et al.*, 2000).

The genes responsible for curli formation are organised into two divergently transcribed operons, *csgBAC* and *csgDEFG*, which are under complex regulation involving numerous regulators, including CsgD, Crl, RpoS, CpxA/R and Rcs (Barnhart & Chapman, 2006). The *csgBAC* operon encodes the main curli structural subunit protein, CsgA, and the nucleator protein, CsgB (Barnhart & Chapman, 2006). CsgA is secreted outside of the cell where CsgB nucleates it into a fibre (Barnhart & Chapman, 2006). CsgC, also encoded on the *csgBAC* operon, has no reported role in curli formation (Barnhart & Chapman, 2006). The *csgDEFG* operon encodes four accessory proteins necessary for curli assembly (Barnhart & Chapman, 2006). CsgD is a positive transcriptional regulator of the *csgBAC* operon. CsgG is an outer membrane lipoprotein which is required for the stability and secretion of CsgA and CsgB, and CsgE and CsgF are putative curli assembly factors, both of which interact with CsgG at the outer membrane (Barnhart & Chapman, 2006).

1.7.3.3 Sfp fimbriae

SF *E. coli* O157:NM strains harbour a cluster of six genes, *sfpA*, *sfpH*, *sfpC*, *sfpD*, *sfpJ* and *sfpG*, on their large plasmid (Brunder *et al.*, 2001, 2006), which encode Sfp fimbriae (sorbitol-fermenting EHEC O157 fimbriae, plasmid encoded) mediating mannose-resistant haemagglutination (Brunder *et al.*, 2001). The possession of *sfp* is a unique characteristic of SF *E. coli* O157:NM strains, that is not present in *E. coli* O157:H7, other *E. coli* or other Enterobacteriaceae (Brunder *et al.*, 2001; Friedrich *et al.*, 2004). It has been shown that Sfp fimbriae are expressed by SF *E. coli* O157:NM strains cultured under anaerobic conditions on solid media simulating the colonic environment, and that the induction of Sfp fimbriae on wild-type SF *E. coli*

O157:NM strains correlated with increased adherence to Caco-2 and HCT-8 cells (Musken *et al.*, 2008).

1.7.4 Plasmid-mediated factors

The majority of VTEC strains of different serotypes are known to contain large plasmids. All strains of *E. coli* O157:H7 possess a highly conserved ~90 kb plasmid, designated pO157, which carries the genes for several putative virulence factors (Nataro & Kaper, 1998). These include the EHEC *hly* operon encoding enterohaemolysin, *espP* which encodes an extracellular serine protease, *katP* encoding a periplasmic catalase-peroxidase, the *etp* gene cluster encoding a complex type II secretion system, and *toxB* which has been linked to type III secretion. However, the role of pO157 in the pathogenesis of *E. coli* O157:H7 is uncertain due to conflicting results from animal experiments (Nataro & Kaper, 1998).

The virulence plasmid of SF *E. coli* O157:NM strains, termed pSFO157, is approximately 32% larger than pO157 at ~120 kb (Brunder *et al.*, 2006). The majority of SF *E. coli* O157:NM strains possess a plasmid which differs from pO157 by the absence of *espP*, *katP* and *toxB*, and the presence of the *sfp* fimbriae gene cluster instead (Bielaszewska *et al.*, 2000; Brunder *et al.*, 1999, 2006; Karch & Bielaszewska, 2001). However, the large plasmids carried by certain SF *E. coli* O157:NM strains isolated in the Czech Republic did not contain any of the putative virulence genes possessed by pO157 (Bielaszewska *et al.*, 2000), suggesting the presence of two different large plasmids in SF *E. coli* O157:NM strains. In contrast to the uniformity of pO157, the plasmids of non-O157 VTEC serotypes are highly variable in both gene composition and arrangement (Brunder *et al.*, 1999).

1.7.4.1 Enterohaemolysin

The *hly* operon encoding enterohaemolysin is found in nearly all *E. coli* O157:H7 strains and is widely distributed among non-O157 VTEC strains (Nataro & Kaper, 1998). Enterohaemolysin belongs to the family of RTX (repeats in toxin) toxins. Enterohaemolysin acts as a pore-forming cytolysin (Schmidt *et al.*, 1995), although its role in pathogenesis is still uncertain. The lysis of erythrocytes *in vivo* would

release haem and haemoglobin, which enhance the growth of *E. coli* O157:H7 and could serve as a source of iron (Nataro & Kaper, 1998). Bonnet *et al*. (1998) suggested that enterohaemolysin may act by lysing eukaryotic cells or by modulating the immune response, thus enhancing virulence, and Aldick *et al*. (2007) demonstrated that enterohaemolysin was cytotoxic to human microvascular endothelial cells, which are the major targets affected during HUS. The possible involvement of enterohaemolysin in the development of HUS is supported by a study which demonstrated that patients infected with *hly*-positive VTEC were at a higher risk of developing HUS than patients infected with *hly*-negative strains (Schmidt & Karch, 1996), and by the fact that patients with HUS had an immune response against enterohaemolysin (Schmidt *et al.*, 1995).

1.7.4.2 EspP

The amino acid sequence of the extracellular serine protease EspP shows homology to several secreted or surface proteins of pathogenic bacteria. These include: PssA, a serine protease encoded on the large plasmid present in bovine VTEC O26:H- strain 413/89-1 (Djafari *et al.*, 1997); EspC, a 110 kDa protein secreted by EPEC; SepA, the major extracellular protein of *Shigella flexneri*; Tsh, a haemagglutinin from an avian-pathogenic *E. coli* strain; and IgA1 proteases from *Neisseria spp.* and *Haemophilus influenzae* (Brunder *et al.*, 1997; Karch *et al.*, 1998).

EspP is capable of cleaving pepsin A and human coagulation factor V (Brunder *et al.*, 1997). Secretion of EspP by VTEC attached to the gastrointestinal mucosa could result in local degradation of coagulation factor V, and increased haemorrhage into the gastrointestinal tract. Thus, EspP could contribute to the bloody diarrhoea seen in many patients suffering from a VTEC infection (Brunder *et al.*, 1997). The importance of EspP in VTEC pathogenesis is supported by the fact that patients suffering from VTEC infections show an antibody response to EspP, whereas healthy control persons do not (Brunder *et al.*, 1997). Similarly, antibodies to PssA were detected in serum from an HUS patient but were absent in serum from a healthy individual (Djafari *et al.*, 1997).

1.7.4.3 KatP

The *katP* gene encodes the bifunctional periplasmic catalase-peroxidase, KatP (Brunder *et al.*, 1996). This enzyme can protect the bacterium against oxidative stress, which is a potential hazard of infecting mammalian cells (Burland *et al.*, 1998).

1.7.4.4 EHEC type II secretion system

A cluster of 13 ORFs, designated *etpC* to *etpO* (EHEC type II pathway), are located on pO157 upstream of the enterohaemolysin operon (Schmidt *et al.*, 1997). This gene cluster has sequence similarities to operons encoding type II secretion systems of Gram negative bacteria, including the pullulanase operons of *Klebsiella pneumoniae* and *K. oxytoca* (Schmidt *et al.*, 1997).

The *etp* gene cluster was detected in 100% of EHEC O157 isolates and in 60% of non-O157 EHEC isolates (serogroups O103, O26 and O111) tested, which had been recovered from clinical cases of infection (Schmidt *et al.*, 1997). In addition, the *etp* genes were only found in 10% of VTEC isolates recovered from bovine faeces and were not detected in EPEC, ETEC, EAEC and EIEC strains (Schmidt *et al.*, 1997).

An example of a protein secreted by the *etp* type II secretion pathway is the metalloprotease, StcE (secreted protease of C1 esterase inhibitor from EHEC).

1.7.4.5 StcE

StcE is encoded by the *stcE* gene located on pO157 and is secreted by the *etp* type II secretion pathway (Lathem *et al.*, 2002). The extracellular levels of StcE are positively regulated by Ler, the LEE-encoded regulator, suggesting that "*stcE* is part of a global virulence regulon central to the pathogenicity of *E. coli* O157:H7" (Lathem *et al.*, 2002). StcE cleaves C1 esterase inhibitor (C1-INH), an essential regulator of the complement system (Lathem *et al.*, 2002), and it has been demonstrated that StcE interaction with C1-INH potentiates rather than destroys the ability of C1-INH to down regulate the classical complement cascade (Lathem *et al.*, 2004). StcE binds to and localises active C1-INH to cell membranes, enhancing the

ability of C1-INH to regulate complement effectors at sites of potential lytic complex formation. The consequence of this is reduced inflammation and complement-mediated lysis at the site of infection (Lathem *et al.*, 2004). In addition, Grys *et al.* (2005) identified glycoprotein 340 and mucin 7 as additional substrates for StcE activity, and reported that StcE reduces the viscosity of human saliva. Furthermore, StcE was shown to contribute to intimate adherence of *E. coli* O157:H7 to HEp-2 cells (Grys *et al.*, 2005). By degrading the protective layer of mucins and glycoproteins on host cells, StcE may impede host clearance of *E. coli* O157 and contribute to the intimate adherence of this pathogen to host cells (Grys *et al.*, 2005). The expression of StcE during human infection has been demonstrated by the presence of StcE antigen in the faeces of a child infected with *E. coli* O157:H7 (Lathem *et al.*, 2002).

1.7.4.6 ToxB

The *toxB* gene is a large ORF, encoding 3169 amino acids, which shows N-terminal homology to large clostridial toxins, including ToxA and ToxB from *Clostridium difficile* (Burland *et al.*, 1998). It has been reported that the deletion of pO157 from an *E. coli* O157:H7 strain reduced adherence to Caco-2 cells, and that introducing the region of pO157 containing *toxB* restored adherence (Tatsuno *et al.*, 2001). However, it was suggested that the contribution of ToxB on the adherence of EHEC to epithelial cells is indirect through the promotion of production and secretion of LEE-encoded type III secreted proteins (Tatsuno *et al.*, 2001).

A ToxB homologue is present in non-O157 EHEC strains, designated Efa1 (EHEC factor for adherence), which is encoded by the chromosomal gene *efa1*. *E. coli* O157:H7 strains lack full-length *efa1* but carry a truncated version of the gene (*efa1'*) on the chromosome (Stevens *et al.*, 2002b), whereas SF *E. coli* O157:NM strains contain a complete *efa1* gene (Janka *et al.*, 2002). Efa1 has been shown to contribute to the adherence of *E. coli* O111:H- to Chinese Hamster Ovary cells (Nicholls *et al.*, 2000). In addition, Efa1 has been identified as an important factor in experimental colonisation of the bovine intestine by EHEC serotypes O5 and O111 (Stevens *et al.*, 2002b), although this may be an indirect consequence of influencing

the expression and secretion of LEE-encoded proteins or other membrane-associated proteins that influence colonisation (Stevens *et al.*, 2002a). However, it has been shown that mutation of *toxB* and *efa1'* did not significantly affect intestinal colonisation by *E. coli* O157:H7 following experimental inoculation in calves or sheep, despite affecting the expression and secretion of LEE-encoded proteins (Stevens *et al.*, 2004).

The *efa1* gene is almost identical in nucleotide sequence to *lifA*, a gene present in EPEC O127:H6 strain E2348/69 that encodes a toxin termed lymphostatin (Klapproth *et al.*, 2000). Lymphostatin inhibits lymphocyte proliferation and IL-2, IL-4 and γ-interferon production (Klapproth *et al.*, 2000), indicating that this toxin, and possibly Efa1, may influence intestinal colonisation by modulating mucosal immunity in the gut (Stevens *et al.*, 2002a).

1.8 Additional factors that contribute to the occurrence of human disease

In addition to the bacterial virulence factors described in section 1.7 it must be remembered that many other factors, including those of host and environment, will influence the outcome of human disease. An illustration of the complexity of interactions is presented in Figure 1.6.

Other pathogen-related characteristics that may contribute to the potential to cause human disease include: the capacity of strains to survive the harsh conditions in the gastrointestinal tract, to resist the immune system and to compete with other organisms in the colonic flora. A further issue that contributes to the human disease outcome is the environmental exposure dose, with greater human exposure possibly resulting in an increased risk of infection. Elements which contribute to exposure dose are: the carriage rate in cattle or other reservoir hosts, shedding levels by cattle, the ability to survive in the environment and undefined differences in animal management, husbandry or human lifestyle that may influence exposure (Chase-Topping *et al.*, 2008; Innocent *et al.*, 2005). Finally, host factors are highly likely to play an important role in the clinical outcome of infection. These include age,

clinical history, or treatment issues such as the administration of antibiotics and/or anti-motility agents.

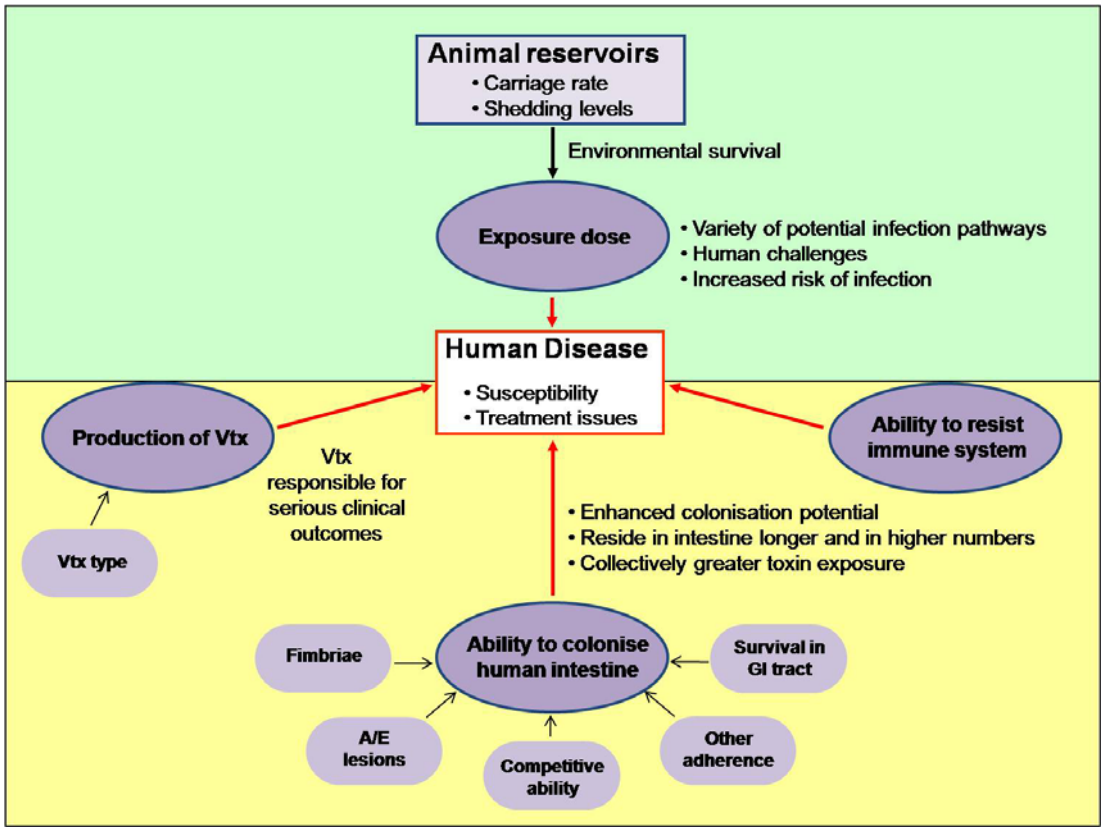


Figure 1.6. Contributing factors for human disease caused by enterohaemorrhagic *E. coli*.

1.9 Research hypothesis and aims

The purpose of this research at the outset was to examine the pathogenic potential of *E. coli* O26. VTEC O26 strains have emerged as significant human pathogens in continental Europe, often causing severe diarrhoea that can progress to HUS. In contrast, human infections associated with *E. coli* O26 are uncommon in Scotland and are generally associated with simple diarrhoea. This is despite the fact that VTEC O26 strains are common and widely dispersed in Scottish cattle. The hypothesis underlying this research is that there are bacterial characteristics that account for the observed differences in human disease. Therefore, the principal aims of this research were to compare *E. coli* O26 isolates from human infections of varying severity to identify bacterial factors which allow these strains to cause more serious disease, and to compare *E. coli* O26 isolates from Scottish cattle to those recovered from human infections, to determine whether strains from the Scottish food-chain represent a threat to human health.

During this research, an outbreak of SF VTEC O157:NM occurred in Scotland, and it appeared that this pathogen was more frequently associated with HUS than the NSF VTEC O157:H7 strains most commonly recognised. This observation posed the question of whether SF VTEC O157:NM strains are more virulent than NSF VTEC O157:H7 strains. Since this question was related to my research interests, it was agreed that my research could be expanded to include SF VTEC O157:NM, with the hypothesis that these pathogens possess virulence traits which contribute to their increased association with HUS.

The common hypothesis underlying this research is that there are fundamental bacterial traits that lead to the observed differences in human disease. The research on these pathogens was to utilise the opportunity of comparing and contrasting characteristics of isolates recovered from different hosts or populations to develop a better understanding of human infection. The thesis is structured in two separate sections with the background information and specific objectives for each pathogen outlined.

Chapter 2

Materials and Methods

2.1 Bacterial strains

All bacterial strains used in this research are detailed in Table 2.1.

2.1.1 Reference *E. coli* strains

All reference *E. coli* strains used in this research are listed in Table 2.1(A). The majority of the reference *E. coli* strains were used as controls in this research. *E. coli* O26 isolate 413/89-1 was recovered from a case of bovine diarrhoea and its LEE pathogenicity island was sequenced during a PhD study by P. Benkel at Justus-Liebig University, Giessen, Germany (GenBank accession no. AJ277443). ZAP078 and ZAP194 are *E. coli* O26 isolates from our laboratory collection of strains. Four well-studied *E. coli* O157:H7 strains were also included as reference strains in this research. NCTC 12900 and Walla-3 are verocytotoxin negative strains of *E. coli* O157:H7. EDL933 is a verocytotoxin positive *E. coli* O157:H7 strain and its genome has been sequenced (Perna *et al.*, 2001). TUV93-0 is a verocytotoxin negative derivative of EDL933. MG1655 is a non-pathogenic *E. coli* K-12 strain and its genome has also been sequenced (Blattner *et al.*, 1997). *E. coli* O127:H6 strain E2348/69 is the prototype typical EPEC strain which has been studied extensively and its genome was recently sequenced (Iguchi *et al.*, 2009).

SF *E. coli* O157:NM strain H8824Δ*csgBA* was created by Ms T. Dransfield (ZAP Laboratory, University of Edinburgh) by deleting the *csgA* and *csgB* genes from SF *E. coli* O157:NM isolate H8824 by allelic exchange. *E. coli* DH5α was used during genetic cloning. DH5α transforms with high efficiency and *rec*A1 and *end*A1 mutations in this strain increase insert stability and improve plasmid DNA yield and quality from minipreps.

2.1.2 *E. coli* O26 isolates

The *E. coli* O26 isolates from different backgrounds that were used in this study are detailed in Table 2.1(B). The information stated about the isolates was provided by their supplier.

2.1.2.1 Bovine *E. coli* O26 isolates

Thirty three *E. coli* O26 isolates recovered from Scottish cattle were obtained from a study conducted to determine the prevalence of non-O157 *E. coli* strains in faeces of Scottish cattle (Pearce *et al.*, 2006). The study by Pearce *et al.* (2006) screened all isolates for the presence of certain virulence determinants and the isolates included in this research were deliberately chosen because they possessed different combinations of virulence genes. Among those selected, the majority carried the gene(s) for verocytotoxin as these are considered to be more pathogenic.

2.1.2.2 Human *E. coli* O26 isolates

Thirty five *E. coli* O26 isolates from cases of human infection with different forms of clinical disease were obtained for this research. Thirteen isolates were recovered from patients in Scotland and were kindly provided by Dr L. Allison (Scottish *E. coli* O157 Reference Laboratory, Scotland). This collection of 13 isolates contains the vast majority of *E. coli* O26 isolates recovered from human infections in Scotland, and most of them were associated with simple diarrhoea.

To investigate *E. coli* O26 isolates associated with more serious clinical disease, it was necessary to obtain isolates from continental Europe. A total of 22 isolates were collected. Sixteen were isolated from patients in Germany and were kindly provided by Prof. H. Karch (Műnster University, Germany), while the remaining six were recovered in Italy and were kindly supplied by Prof. A. Caprioli (Istituto Superiore di Sanità, Rome, Italy). The isolates obtained from continental Europe had been previously screened for the presence of verocytotoxin genes and were specifically selected for this study because they possessed various combinations of verocytotoxin gene carriage and were associated with different clinical forms of disease, including life-threatening HUS.

2.1.3 *E. coli* O157 isolates

The *E. coli* O157 isolates used in this study are detailed in Table 2.1(C). Wild-type sorbitol-fermenting (SF) VTEC O157:NM isolates H8824, H8432, H8489, H8757 and H8478 were recovered during the 2006 Scottish outbreak cluster and shared an

indistinguishable PFGE profile. A single SF VTEC O157:NM isolate (H2687) was obtained from an isolated case in Scotland (Locking *et al.*, 2004) and was associated with bloody diarrhoea. Four SF VTEC O157:NM isolates collected in Germany (340/97, 080/01, E02/879 and E06/486) were also included in this study and were kindly provided by Prof. H. Karch. Four non-sorbitol-fermenting (NSF) VTEC O157:H7 strains were included for comparison in this research. Three (H77, H511 and 1477/AI) were isolated from separate Scottish outbreaks (Cowden, 1997; Outbreak Control Team, 2001; SCIEH, 2001) and the remaining NSF VTEC O157:H7 isolate was the sequenced strain EDL933 (Perna *et al.*, 2001). The VTEC O157 isolates recovered in Scotland were kindly provided by Dr L. Allison.

Table 2.1. Bacterial strains. (A) Reference *E. coli* strains (B) *E. coli* O26 isolates (C) *E. coli* O157 isolates.

(A)

Strain (original designation)	**Serotype**	**Description**	**Source or reference**
413/89-1	O26:H-	Isolated from a case of bovine diarrhoea	T. Chakraborty[a]
ZAP078	O26:$H_{untyped}$	Isolated from a case of human disease	Reference Laboratory, Aberdeen[b]
ZAP194	O26:K60:$H_{untyped}$	Produces A/E lesions in cattle	Pearson *et al.* (1999)
NCTC 12900	O157:H7	Verocytotoxin negative strain of *E. coli* O157:H7	NCTC, HPA[c]
Walla-3	O157:H7	Verocytotoxin negative strain of *E. coli* O157:H7	Ostroff *et al.* (1990)
EDL933	O157:H7	Sequenced *E. coli* O157:H7	Perna *et al.* (2001)
TUV93-0	O157:H7	Verocytotoxin negative derivative of EDL933	Campellone *et al.* (2002)
MG1655	K-12	Sequenced *E. coli* K-12	Blattner *et al.* (1997)
E2348/69	O127:H6	Sequenced EPEC strain	Iguchi *et al.* (2009)
H8824Δ*csgBA*	SF O157:NM	SF VTEC O157:NM isolate H8824 with *csgBA* deleted	ZAP laboratory strain
DH5α	K-12	Used in genetic cloning	Laboratory stock

[a] T. Chakraborty, Justus-Liebig University, Giessen, Germany.
[b] Scottish National Reference Laboratory for *Campylobacter* and *E. coli* O157, Aberdeen, Scotland.
[c] NCTC, Health Protection Agency, Colindale, London, UK.

Table 2.1. Bacterial strains *continued*

(B)

Origin of isolate	Isolate[a] (original designation)	Serotype[b]	Clinical information[b,c]	Date of isolation[b]	Source or reference
Bovine (Scotland)	ZAP1077_B1 (WX007861S01K)	O26:H11		12/06/2002	Pearce *et al*. (2006)
	ZAP1078_B2 (WX007869S01K)	O26:H11		12/06/2002	
	ZAP1079_B3 (WX008325S01K)	O26:H11		22/07/2002	
	ZAP1080_B4 (WX008326S01K)	O26:H11		22/07/2002	
	ZAP1081_B5 (WX008349S01K)	O26:H11		22/07/2002	
	ZAP1082_B6 (WX008576S01K)	O26:H11		29/07/2002	
	ZAP1083_B7 (WX008650S01K)	O26:H11		12/08/2002	
	ZAP1084_B8 (WX008796S01K)	O26:H11		19/08/2002	
	ZAP1085_B9 (WX008809S01K)	O26:H11		19/08/2002	
	ZAP1086_B10 (WX008821S01K)	O26:H11		19/08/2002	
	ZAP1087_B11 (WX008859S01K)	O26:H11		19/08/2002	
	ZAP1088_B12 (WX009926S01K)	O26:H11		16/09/2002	
	ZAP1089_B13 (WX009931S01K)	O26:H11		16/09/2002	
	ZAP1090_B14 (WX010765S01K)	O26:H11		07/10/2002	
	ZAP1091_B15 (WX011378S01K)	O26:H11		11/11/2002	
	ZAP1092_B16 (WX011379S01K)	O26:H11		11/11/2002	
	ZAP1093_B17 (WX012116S01K)	O26:H11		09/12/2002	
	ZAP1094_B18 (WX014441S01K)	O26:H11		28/04/2003	
	ZAP1095_B19 (WX014851S01K)	O26:H11		13/05/2003	
	ZAP1096_B20 (WX016290S01K)	O26:H11		30/06/2003	
	ZAP1097_B21 (WX016693S01K)	O26:H11		28/07/2003	
	ZAP1098_B22 (WX016732S01K)	O26:H11		28/07/2003	
	ZAP1099_B23 (WX017042S01K)	O26:H11		04/08/2003	
	ZAP1100_B24 (WX017873S01K)	O26:$H_{untyped}$		25/08/2003	

Table 2.1(B) ***continued***

Origin of isolate	Isolate[a] (original designation)	Serotype[b]	Clinical information[b,c]	Date of isolation[b]	Source or reference
Bovine (Scotland)	ZAP1101_B25 (WX017876S01K)	O26:$H_{untyped}$		25/08/2003	Pearce *et al*. (2006)
	ZAP1102_B26 (WX018178S01K)	O26:H11		08/09/2003	
	ZAP1103_B27 (WX019091S01K)	O26:H11		20/10/2003	
	ZAP1104_B28 (WX019199S01K)	O26:H11		27/10/2003	
	ZAP1105_B29 (WX019482S01K)	O26:H11		10/11/2003	
	ZAP1106_B30 (WX019909S01K)	O26:$H_{untyped}$		10/12/2003	
	ZAP1107_B31 (WX020495S01K)	O26:H11		12/01/2004	
	ZAP1108_B32 (WX020561S01K)	O26:H11		19/01/2004	
	ZAP1109_B33 (WX020761S01K)	O26:H11		26/01/2004	
Human (Scotland)	ZAP1110_S1 (972)	O26:$H_{untyped}$	Diarrhoea		L. Allison,
	ZAP1111_S2 (1003)	O26:$H_{untyped}$	No info		Scottish *E. coli* O157
	ZAP1112_S3 (1122)	O26:$H_{untyped}$	Diarrhoea		Reference Laboratory,
	ZAP1113_S4 (1768)	O26:$H_{untyped}$	Diarrhoea		Scotland
	ZAP1114_S6 (2261)	O26:$H_{untyped}$	Diarrhoea		
	ZAP1115_S7 (2410)	O26:$H_{untyped}$	Diarrhoea		
	ZAP1116_S8 (2444)	O26:$H_{untyped}$	BD		
	ZAP1117_S9 (2820)	O26:$H_{untyped}$	Diarrhoea		
	ZAP1118_S10 (2848)	O26:$H_{untyped}$	Diarrhoea		
	ZAP1119_S11 (4396)	O26:$H_{untyped}$	Diarrhoea		
	ZAP1120_S12 (5606)	O26:$H_{untyped}$	Diarrhoea		
	ZAP1152_S35 (H7788)	O26:$H_{untyped}$	Diarrhoea		
	ZAP1153_S36 (H7291)	O26:$H_{untyped}$	BD		

Table 2.1(B) ***continued***

Origin of isolate	Isolate[a] (original designation)	Serotype[b]	Clinical information[b,c]	Date of isolation[b]	Source or reference
Human (Germany)	ZAP1121_G13 (1616/96)	O26:H-	Diarrhoea	1996	H. Karch,
	ZAP1122_G14 (2150/98)	O26:H-	HUS	1998	Műnster University,
	ZAP1123_G15 (2185/99)	O26:H11	HUS	1999	Germany
	ZAP1124_G16 (2245/98)	O26:H-	HUS	1998	
	ZAP1125_G17 (2514/99)	O26:H-	Diarrhoea	1999	
	ZAP1126_G18 (2640/99)	O26:H11	HUS	1999	
	ZAP1127_G19 (2959/99)	O26:H-	Diarrhoea	1999	
	ZAP1128_G20 (2983/00)	O26:H11	HUS	2000	
	ZAP1129_G21 (3073/00)	O26:H11	HUS	2000	
	ZAP1130_G22 (3901/97)	O26:H-	HUS	1997	
	ZAP1131_G23 (4166/94)	O26:H-	Diarrhoea	1994	
	ZAP1132_G24 (4497/00)	O26:H-	HUS	2000	
	ZAP1133_G25 (4602/97)	O26:H-	Diarrhoea	1997	
	ZAP1134_G26 (5720/96)	O26:H-	HUS	1996	
	ZAP1135_G27 (6932/94)	O26:H11	Diarrhoea	1994	
	ZAP1136_G28 (8574/96)	O26:H11	Diarrhoea	1996	
Human (Italy)	ZAP1146_I29 (ED 17)	O26:$H_{untyped}$	HUS		A. Caprioli,
	ZAP1147_I30 (ED 147)	O26:$H_{untyped}$	HC		Istituto Superiore di
	ZAP1148_I31 (ED 166)	O26:$H_{untyped}$	HUS		Sanità, Rome, Italy
	ZAP1149_I32 (ED 180)	O26:$H_{untyped}$	HUS		
	ZAP1150_I33 (ED 551)	O26:$H_{untyped}$	HUS		
	ZAP1151_I34 (ED 567)	O26:$H_{untyped}$	HUS		

[a] The suffix indicates the origin of the isolate: B, bovine; S, human (Scotland); G, human (Germany); I, human (Italy).
[b] Information provided by the supplier of the isolate.
[c] BD, bloody diarrhoea; HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome.

Table 2.1. Bacterial strains *continued*

(C)

Serotype[a]	Isolate(s)	Country of origin	Source or reference
NSF VTEC O157:H7	H77	Scotland	Outbreak Control Team (2001)
	H511	Scotland	SCIEH (2001)
	1477/AI	Scotland	Cowden (1997)
SF VTEC O157:NM	H2687	Scotland	Locking *et al.* (2004)
	H8824, H8432, H8489, H8757, H8478	Scotland	2006 Scottish outbreak cluster
	340/97, 080/01, E02/879, E06/486	Germany	H. Karch, Műnster University, Germany

[a] NSF, non-sorbitol-fermenting; SF, sorbitol-fermenting.

2.2 Bacterial culture conditions, media and chemicals

All chemicals used were from Sigma-Aldrich unless otherwise stated. All bacterial strains were stored on Protect beads (Technical Service Consultants Ltd) at -70°C. Strains were routinely cultured at 37°C in Luria-Bertani (LB) broth (Melford) or on LB agar (Formedium), prepared according to manufacturer's instructions. The ability of VTEC O157 isolates recovered in Scotland to ferment sorbitol was confirmed by plating on sorbitol MacConkey agar (Merck), prepared according to manufacturer's instructions. In Vero cell cytotoxicity assays and for secreted protein analysis, Dulbecco's modified eagle medium with 25mM HEPES (DMEM-HEPES; Gibco) was used to culture *E. coli* O26 isolates while minimal essential medium with 25mM HEPES (MEM-HEPES; Sigma-Aldrich), supplemented with glucose (final concentration of 0.2%) and 0.25 μM $Fe(NO_3)_3$, was used to culture *E. coli* O157 isolates. In adherence assays, bacteria were cultured on CFA agar plates and in CDMT broth (see Appendix 1 for recipes). LB, CFA and CDMT broths were used to culture bacteria in biofilm assays. To screen for surface curli expression bacteria were cultured on CFA-Congo red (CR) indicator plates (0.01% CR).

2.3 Cell culture conditions

Vero (African green monkey kidney) cells were grown in Eagle minimum essential medium with Earle's salts (Sigma-Aldrich) supplemented with 10% (v/v) foetal bovine serum (FBS, Sigma-Aldrich), 1% non-essential amino acids (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco). The Vero cells were a kind gift from Karla Sanchez and the cells used in experiments had been sub-cultured between 27 and 35 times. Caco-2 cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% (v/v) FBS, 1% non-essential amino acids, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The Caco-2 cells were also kindly provided by Karla Sanchez and the cells used in experiments had been sub-cultured between 15 and 25 times. All cultivations were performed at 37ºC in 5% CO_2.

2.4 DNA analysis and genetic manipulation methods

2.4.1 Genomic DNA extraction from *E. coli* isolates

Genomic DNA was extracted from *E. coli* isolates using the ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen), according to manufacturer's instructions for Gram negative bacteria. Aliquots of purified DNA were stored at -20°C. Genomic DNA was diluted 1:500 in MQ water (Sigma-Aldrich) before used as template DNA in PCR reactions.

2.4.2 Preparation of crude DNA extracts from *E. coli* isolates

Crude DNA preparations were used as templates in PCR screens. A bacterial colony from overnight growth on LB agar was suspended in 100 µl sterile phosphate-buffered saline (PBS) and boiled for 15 min in a heated block. Aliquots of bacterial lysate were stored at -20°C and 1 µl of lysate was used as DNA template in PCR reactions.

2.4.3 Plasmid DNA extraction from *E. coli* strains

Plasmids were extracted from bacteria, cultured overnight in 5 ml LB broth supplemented with the appropriate antibiotic, using the PureLink HiPure plasmid miniprep kit (Invitrogen).

2.4.4 DNA amplification by polymerase chain reaction (PCR)

PCRs were typically carried out in sterile thin-wall tubes in 50 µl reaction volumes. Generally, PCRs contained 1 µl template DNA, 200 µM dNTP mixture (Roche), 1 µM each primer (Table 2.2), 1 x ThermoPol *Taq* reaction buffer [20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 at 25°C] (NEB), 2 U *Taq* DNA polymerase (NEB) and MQ water to the final volume. Some of the virulence gene screening PCRs were carried out using Platinum Blue PCR SuperMix (Invitrogen), according to manufacturer's instructions, and contained 1 µl template DNA and 1 µM each primer. The genes for MLST (section 2.4.10) and the vtx_2 genes for sequencing were amplified by PCR using a *Taq* PCR core kit (Qiagen), according to manufacturer's instructions. These PCR reactions contained 2 µl template DNA, 200 µM each dNTP, 0.2 µM each primer, 1 x PCR buffer, 1 x Q-

solution, 2.5 U *Taq* DNA polymerase and MQ water to the final volume. For sequence analysis of the region required for curli formation, high-fidelity amplification of DNA template was achieved using iProof high-fidelity DNA polymerase (Bio-Rad), according to manufacturer's instructions. These reactions contained 1 µl template DNA, 200 µM each dNTP, 0.5 µM each primer, 1 x HF buffer (Bio-Rad), 0.4 U iProof DNA polymerase and MQ water to the final volume. iProof DNA polymerase comprises a unique *Pyrococcus*-like proofreading enzyme fused to a dsDNA binding protein, Sso7d, and is more accurate than *Taq* DNA polymerase.

All PCRs were performed in a Thermo-Hybaid PCR Express cycler and the cycling conditions for each PCR are detailed in Table 2.2. Amplification products were resolved by agarose gel electrophoresis, as described in section 2.4.5. DNA negative PCR controls, which contained all reagents except template DNA, were performed in every PCR run and all reactions were repeated if the negative controls contained amplification products. Positive and negative controls were also performed in every PCR screen using template DNA from positive and negative reference *E. coli* strains. Test strains were regarded as positive if they produced amplicons of the correct size and negative if they failed to produce amplification products in two independent PCR repeats.

Table 2.2. Primers and PCR conditions. The primer name and sequence are listed, together with the PCR conditions for each primer pair. The table is sectioned to group primers used for similar purposes: top – characterisation PCRs; second – MLST; third – DNA sequencing; bottom – amplification of promoter regions for cloning purposes.

Primer name	Primer sequence (5'→3')[a]	PCR conditions[b]			Amplicon size (bp)	Reference
		Denaturing	Annealing	Extension		
O26wzxF O26wzxR	GCGCTGCAATTGCTTATGTA TTTCCCCGCAATTTATTCAG	94°C, 30 s	54°C, 45 s	72°C, 30 s	152	DebRoy *et al*. (2004)
O26wzyF O26wzyR	TAAATTGCGGGGAAAGAATG GACTTCATGGGTACCGCCTA	94°C, 30 s	60°C, 45 s	72°C, 30 s	276	"
vtx1F vtx1R	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	94°C, 30 s	60°C, 45 s	72°C, 30 s	180	Paton & Paton (1998)
vtx2F vtx2R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	94°C, 30 s	60°C, 45 s	72°C, 30 s	255	"
eaeAF eaeAR	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	94°C, 30 s	60°C, 45 s	72°C, 30 s	384	"
hlyAF hlyAR	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	94°C, 30 s	60°C, 45 s	72°C, 30 s	534	"
sepLF sepLR	GCTAAGCCTGGGATATCGC ACAATCGATACCCGAGAAGG	94°C, 30 s	60°C, 45 s	72°C, 60 s	725	This study
cad1 LS1	TGGACGGAGTAACAAGCC ATCCTCATTGCGTGGCTG	94°C, 30 s	52°C, 60 s	72°C, 120 s	947	P. Benkel[c]

Table 2.2 ***continued***

Primer name	Primer sequence (5'→3')[a]	PCR conditions[b]			Amplicon size (bp)	Reference
		Denaturing	Annealing	Extension		
espB73 espB52	TTCAGCATAACGGCAAGC TTAGCCAACAGATAGATG	94°C, 30 s	52°C, 60 s	72°C, 120 s	1480	P. Benkel[c]
univ tccP/tccP2-F tccP-R	GTAAAAACCAGCTCACCTTTTTC TCACGAGCGCTTAGATGTATTAAT	94°C, 60 s	64°C, 60 s	72°C, 90 s	variable	Kozub-Witkowski *et al*. (2008)
tccP-F	ATGATTAACAATGTTTCTTCACTT	94°C, 60 s	54°C, 60 s	72°C, 90 s	variable	Garmendia *et al*.(2005b)
tccP2-F	ATGATAAATAGCATTAATTCTTT	94°C, 60 s	54°C, 60 s	72°C, 90 s	variable	Ogura *et al*. (2007)
tirY474-F tir-R	CATATTTATGATGAGGTCGCTC TAAAAGTTCAGATCTTGATGACAT	94°C, 30 s	52°C, 60 s	72°C, 60 s	401	"
tirS478-F	TCTGTTCAGAATATGGGGAATA	94°C, 30 s	58°C, 60 s	72°C, 60 s	407	"
FLIC1F FLIC2R	ATGGCACAAGTCATTAATACCCAAC CTAACCCTGCAGCAGAGACA	94°C, 30 s	60°C, 60 s	72°C, 120 s	1758	Fields *et al.* (1997)
STS-1 CON-2	CTCAAGCATAAAAATTTTAAACTAACTG ACGTCCCTGAACCTGGGTAGGTTA	94°C, 60 s	62°C, 60 s	72°C, 60 s	943	Li *et al.* (1997)
2535 3137	GCCGGATTATGGGAAAGA GCCGCTGTAGAACTGAGG	94°C, 45 s	50.5°C, 45 s	72°C, 90 s	603	Holden *et al*. (2006)
adkF adkR	ATTCTGCTTGGCGCTCCGGG CCGTCAACTTTCGCGTATTT	94°C, 60 s	54°C, 60 s	72°C, 120 s	583	*E. coli* MLST website[d]

Table 2.2 ***continued***

Primer name	Primer sequence (5'→3')[a]	PCR conditions[b]			Amplicon size (bp)	Reference
		Denaturing	Annealing	Extension		
fumCF fumCR	TCACAGGTCGCCAGCGCTTC GTACGCAGCGAAAAAGATTC	94°C, 60 s	54°C, 60 s	72°C, 120 s	806	*E. coli* MLST website[d]
gyrBF gyrBR	TCGGCGACACGGATGACGGC ATCAGGCCTTCACGCGCATC	94°C, 60 s	60°C, 60 s	72°C, 120 s	911	"
icdF icdR	ATGGAAAGTAAAGTAGTTGTTCCGGCACA GGACGCAGCAGGATCTGTT	94°C, 60 s	54°C, 60 s	72°C, 120 s	878	"
mdhF mdhR	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	94°C, 60 s	60°C, 60 s	72°C, 120 s	932	"
purAF purAR	CGCGCTGATGAAAGAGATGA CATACGGTAAGCCACGCAGA	94°C, 60 s	54°C, 60 s	72°C, 120 s	816	"
recAF recAR	CGCATTCGCTTTACCCTGACC TCGTCGAAATCTACGGACCGGA	94°C, 60 s	58°C, 60 s	72°C, 120 s	780	"
espAF espAR	CCTTCTCGGGTATCGATTGTCG CAGAGGGCGTCACTAATGAGTG	94°C, 60 s	58°C, 60 s	72°C, 120 s	1012	This study
LEE1promF LEE1promR	CGAATGGTACGGTTATGCGGG GCTCTCGCAGTCGCTTTGCTTCC	94°C, 60 s	58°C, 60 s	72°C, 120 s	645	This study
vtx2Aseq_F1 vtx2Aseq_R2	TACCAGGCTCGCTTTTGCGG CGCCATTGCATTAACAGAAGC	94°C, 30s	60°C, 45s	72°C, 80s	1110	This study

Table 2.2 ***continued***

Primer name	Primer sequence (5'→3')[a]	PCR conditions[b]			Amplicon size (bp)	Reference
		Denaturing	Annealing	Extension		
vtx2Aseq_F2	CCATGACAACGGACAGCAG					This study
vtx2Aseq_R1	CTGTATCTGCCTGAAGCGTAAGGC					This study
vtx2Bseq_F	CCAGAATGTCAGATAACTGGC	94°C, 30s	58°C, 45s	72°C, 30s	476	This study
vtx2Bseq_R	GGCAACTGTCAACTGACTG					
curli1F	CGCTTAAACAGTAAATGCCG	98°C, 10s[e]	60°C, 30s[e]	72°C, 35s[e]	1046	This study
curli1R	CCGCATGGTGACCAACGA					
curli2F	TTCCTTATGAAGCTGGGGC	98°C, 10s[e]	60°C, 30s[e]	72°C, 35s[e]	1054	This study
curli2R	CGGAATCAGCCCTCCTTAC					
curli3F	CGCTGATGAACAACGAACG	98°C, 10s[e]	60°C, 30s[e]	72°C, 35s[e]	1028	This study
curli3R	CCCGTCGCTGATTGCTGC					
curli4F	CTCCACACCACCGTGGAC	98°C, 10s[e]	60°C, 30s[e]	72°C, 35s[e]	1047	This study
curli4R	GCTTGCAGAGCAAGTGCAG					
curli5F	GGCAGGTGTTGTTCCTCAG	98°C, 10s[e]	60°C, 30s[e]	72°C, 35s[e]	886	This study
curli5R	CCTTGAGGGTTGTGTTATCC					
T3	AATTAACCCTCACTAAAGGGAA					
T7	GTAATACGACTCACTATAGG					
sfpprom_F	cgcggatccATCAACGCCAAATGTGTTTAC	94°C, 15 s	60°C, 30 s	72°C, 60 s	800	This study
sfpprom_R	cggggtaccAATCAAACCGAGAATTGTCTT					

Table 2.2 ***continued***

Primer name	Primer sequence (5'→3')[a]	PCR conditions[b]			Amplicon size (bp)	Reference
		Denaturing	Annealing	Extension		
csgBACprom_F	cgcggatccGTTGTACATTTGGTTTTTATTGCAC	94°C, 15 s	60°C, 30 s	72°C, 60 s	524	This study
csgBACprom_R	cggggtaccCAATTTGTTTTTCATGTTGTCACC					

[a] The lowercase portions of some sequences are non-matching and the underlined bases show the incorporation of restriction sites.
[b] Unless otherwise stated, PCR cycling conditions included an initial denaturing step of 94°C for 4 min, and after 30 cycles a final extension step of 72°C for 10 min was performed.
[c] Primer sequences were designed during a PhD study by P. Benkel at Justus-Liebig University, Giessen, Germany.
[d] *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli).
[e] PCR cycling conditions included an initial denaturing step of 98°C for 30 sec, and after 35 cycles a final extension step of 72°C for 10 min was performed.

2.4.5 DNA electrophoresis

DNA fragments were resolved by electrophoresis in 1% (w/v) agarose gels in 1 x Tris-borate/EDTA (TBE) buffer [89 mM Tris-borate and 2 mM EDTA, pH 8.3 (Sigma-Aldrich)], containing ethidium bromide or SafeView (NBS Biologicals) for DNA staining. Electrophoresis was performed in 1 x TBE buffer at constant voltage (100 V) using Bio-Rad systems. DNA samples were mixed with DNA loading buffer (Invitrogen) before loading and fragment sizes were determined by comparison to molecular size markers [1 kb or 100 bp (Invitrogen)]. DNA fragment visualisation was achieved under UV illumination in a Flowgen MultiImage light cabinet (Shenstone, England) and images were captured using ChemiImager 4000i v.4.04 software.

2.4.6 DNA fragment purification

2.4.6.1 Column and gel purification

DNA fragments from PCR and restriction digests were purified directly using the PureLink PCR purification kit (Invitrogen), or were purified from agarose gels using the PureLink Quick gel extraction kit (Invitrogen), following manufacturer's instructions in both cases.

2.4.6.2 PEG purification

Amplification products from MLST PCRs were purified using the polyethylene glycol (PEG) method. This is a simple, cost-effective method for purifying DNA fragments following PCR amplification in 96-well plates. Briefly, 60 µl 20% PEG/2.5 M NaCl solution was added to each well of the PCR plate containing 50 µl PCR reaction. The plate was sealed tightly with adhesive film and the contents of the wells mixed using a vortex mixer. The plate was centrifuged briefly at 500 x *g* to ensure the contents were at the bottom of the wells before it was incubated at room temperature for 30 min. PCR products were pelleted by centrifugation (2,750 x *g*, 1 h at 4ºC) and the supernatants were discarded by gently inverting the plate and centrifuging it (500 x *g*, 1 min at 4ºC) upside down on tissues. The DNA pellets were washed twice by adding 150 µl cold 70% ethanol and centrifuging (2,750 x *g*,

10 min at 4ºC). Supernatants were discarded by centrifuging inverted as before. PCR pellets were resuspended in 50 µl MQ water.

2.4.7 Characterisation PCRs

The primer sequences and cycling conditions for the PCRs are detailed in Table 2.2. The presence of the O26 *wzx* (O-unit flippase) and O26 *wzy* (O-unit polymerase) genes were detected by PCR, using primer pairs O26wzxF and O26wzxR and O26wzyF and O26wzyR, respectively, as previously described (DebRoy *et al.*, 2004). The detection of genes encoding verocytotoxin 1 (vtx_1), verocytotoxin 2 (vtx_2), intimin (*eae*) and enterohaemolysin (*hlyA*) was performed using the multiplex PCR developed by Paton and Paton (1998). The primer sequences were the same as those described previously (Paton & Paton, 1998) but the PCR cycling conditions were simplified for this research. The possession of the LEE pathogenicity island was confirmed by PCR screening for *sepL* (SepL) using primer pair sepLF and sepLR. The *pheU* tRNA gene was screened for the presence of the LEE by PCR using three different primer pair combinations. Primer pair cad1 and LS1 amplified the left junction of the LEE inserted into *pheU* and primer pair espB73 and espB52 amplified the right junction of the LEE inserted into *pheU*. Intact *pheU* was detected by PCR screening with primer pair cad1 and espB52 and produced a 1.26 kb amplicon. The detection of genes encoding TccP (*tccP*) and TccP2 (*tccP2*) was performed by PCR using a universal primer pair, univ tccP/tccP2-F and tccP-R. The specific detection of *tccP* and *tccP2* was achieved by PCR amplification using forward, gene-specific primers (tccP-F and tccP2-F, respectively) with a conserved reverse primer (tccP-R). Forward, gene-specific primers (tirY474-F, tirS478-F) were used with a conserved reverse primer (tir-R) to discriminate between $tir_{E2348/69}$ and tir_{EDL933} gene types. PCR to detect the presence of the 16 bp deletion in the *fim* switch that controls type 1 fimbriae expression was performed using primer pair STS-1 and CON-2, as previously described (Li *et al.*, 1997).

2.4.8 H-typing by *fliC* PCR-restriction fragment length polymorphism (RFLP)

The gene encoding the flagellin subunit (*fliC*) was detected and characterised using the *fliC* PCR-RFLP method described previously (Fields *et al.*, 1997). Briefly, the

fliC gene was amplified by PCR using primer pair FLIC1F and FLIC2R (Table 2.2). The purified PCR product (5 µl) was digested with *Rsa*I (NEB) in a 10 µl reaction, according to manufacturer's instructions. Digestion was performed at 37°C for 2 h and the restriction fragments were separated on a 2% agarose gel.

2.4.9 *fim* switch orientation assay

Orientation of the *fim* switch was carried out as described previously (Holden *et al.*, 2006; Leathart & Gally, 1998; Roe *et al.*, 2001). Briefly, PCR using primers 2535 and 3137 (Table 2.2) was used to amplify a 603 bp region that incorporates the invertible *fimA* promoter element. Template DNA for the PCR was prepared by adding 50 µl of bacterial culture (following 3 days of consecutive subculture in LB broth) or a colony from cultures grown on CFA agar to 50 µl MQ water and boiling at 100°C for 15 min. The PCR-amplified product (4 µl) was digested asymmetrically with *Hinf*I (NEB) in a 10 µl reaction at 37°C for 1 h. The digests were mixed with 10 x loading buffer (65% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol) and DNA fragments were separated by electrophoresis in a 4% polyacrylamide TBE gel [4% acrylamide-bisacrylamide, 0.1% ammonium persulphate (APS), 0.001% N,N,N',N'-tetramethylethylenediamine (TEMED) in 1 x TBE buffer]. Electrophoresis was performed in 1 x TBE buffer at constant voltage (160 V) for 2 h 30 min using a Hoefer SE600 Midi gel electrophoresis unit. Fragments were visualised after staining with ethidium bromide.

2.4.10 Multilocus sequence typing (MLST)

MLST was performed using a published scheme for *E. coli* (Wirth *et al.*, 2006). The nucleotide sequences of internal fragments of the following seven housekeeping genes (protein products are shown in parentheses) were determined: *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif). Also sequenced were the *espA* gene (EspA) and the region upstream of *LEE1*, likely to contain the promoter sequence. The gene fragments were amplified from genomic DNA from the *E. coli* O26 isolates by PCR using a *Taq* PCR core kit (Qiagen) and

the primer pairs detailed in Table 2.2. The primers used to amplify *espA* and the region upstream of *LEE1* were designed using sequence data for *E. coli* O26 isolate 413/89-1 (GenBank accession no. AJ277443). The initial 'high-throughput' runs of PCR reactions were performed in 96-well plates and products were purified using the PEG method (section 2.4.6.2). Unsuccessful PCR or sequencing reactions were repeated and, in these instances, PCR was performed in thin-wall tubes and amplicons were purified using the PureLink PCR purification kit (Invitrogen), following manufacturer's instructions. PCR products were sequenced directly with the PCR primers (section 2.4.11). The sequences obtained for the housekeeping genes were assigned allele numbers in accordance with the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli). Novel alleles discovered were confirmed by repeating the PCR and sequencing reactions before they were submitted to the *E. coli* MLST database. The sequence type (ST) and ST complex of each isolate was designated in accordance with the *E. coli* MLST website. Sequence data for *espA* and the region upstream of *LEE1* were compared using ClustalX. Each unique sequence at each loci was given a different allele number and allele numbers were assigned in the order in which they were discovered.

2.4.11 DNA sequencing

Nucleotide sequencing for MLST purposes was performed by the zoology sequencing service, University of Oxford. All remaining DNA sequencing in this research was carried out by the GenePool, School of Biological Sciences, University of Edinburgh and was performed using an ABI Prism BigDye terminator cycle sequencing kit version 3.1 (Applied Biosystems), and reactions were analysed on an ABI 3730 DNA sequencer.

The nucleotide sequences of vtx_2 from the six Scottish SF VTEC O157:NM isolates were determined following PCR amplification of $vtxA_2$ and $vtxB_2$ using a *taq* PCR core kit (Qiagen) and primer pairs vtx2Aseq_F1 and vtx2Aseq_R2 and vtx2Bseq_F and vtx2Bseq_R, respectively (Table 2.2). These primers were designed using sequence data for *E. coli* O157:H7 EDL933 (GenBank accession no. NC_002655). The amplicons were purified and sequenced directly with the PCR primers and

internal *vtxA*$_2$ primers vtx2Aseq_F2 and vtx2Aseq_R1 (Table 2.2). For sequence analysis of the region required for curli formation (*csgBAC* and *csgDEFG* operons and the *csgB*-to-*csgD* intergenic region), the entire region was amplified with five separate PCRs using iProof high-fidelity DNA Polymerase (Bio-Rad) and primer pairs curli1F and curli1R, curli2F and curli2R, curli3F and curli3R, curli4F and curli4R and curli5F and curli5R (Table 2.2). PCR products were purified, cloned into the StrataClone PCR cloning vector, pSC-B, with a StrataClone Blunt PCR cloning kit (Stratagene), and sequenced with primers T3 and T7.

2.4.12 DNA sequence analysis

A double-stranded DNA sequence for the region of interest was assembled using the Staden package release v1.6.0. Briefly, DNA sequencing chromatogram files were prepared for assembly and analysis with pregap4 and were then assembled into contigs using gap4. Sequence data was then viewed and edited using gap4. Assembled DNA sequences were aligned and compared using ClustalX 1.8 (Thompson *et al.*, 1997) and alignments were annotated using GeneDoc version 2.5.

2.4.13 Construction of plasmid-based promoter-GFP fusions

The promoters for *sfpA* (Sfp fimbriae main subunit) and *csgBAC* (curli main subunit) were amplified by PCR from isolate H8824 using primer pairs sfpprom_F and sfpprom_R and csgBACprom_F and csgBACprom_R, respectively (Table 2.2). These primers incorporated *Bam*HI (sfpprom_F, csgBACprom_F) and *Kpn*I (sfpprom_R, csgBACprom_R) sites at their termini and allowed the promoters to be cloned in frame to *gfp* in pAJR70 (Roe *et al.*, 2003), creating translational fusions. Briefly, the PCR-amplified products were gel purified and cloned into pSC-B with a StrataClone Blunt PCR cloning kit. The nucleotide sequences of the amplified promoters were confirmed by sequencing. Vector and insert DNA were prepared for cloning by digesting pAJR70 and pSC-B containing the promoter of interest sequentially with *Bam*HI (Roche) and *Kpn*I (Roche), according to manufacturer's instructions. Linearised pAJR70 was treated with shrimp alkaline phosphatase (NEB) to dephosphorylate 5' restricted termini and prevent vector re-ligation. Following gel purification, vector (1 µl) and insert DNA (7 µl) were mixed with 1 µl

T4 DNA ligase (NEB) and 1 µl 10 x ligase reaction buffer (NEB) [final concentration: 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, pH 7.5 at 25°C] and incubated at room temperature for 3 h 30 min to allow ligation. The promoter for *csgD* (positive transcriptional regulator of the *csgBAC* operon) cloned into pAJR70 was constructed previously (Low *et al.*, 2006).

2.4.14 Transformation of competent *E. coli*

E. coli strains were made competent for transformation with plasmid DNA using the methods described in Sambrook *et al*. (1989).

2.4.14.1 Preparation and transformation of chemically-competent *E. coli* cells

E. coli K-12 DH5α was cultured in 5 ml LB broth with shaking (200 rpm) at 37ºC overnight. The following day the overnight bacterial culture was diluted 1:100 in LB broth and cultured under the same conditions to an optical density at 600 nm (OD_{600}) of 0.5 – 0.7. The culture was chilled on ice for 30 min before it was centrifuged (4,000 x *g*, 8 min at 4ºC). Cell pellets were gently suspended in 0.4 volumes of ice-cold transformation buffer I (TFB I, see Appendix 1) and kept on ice for 10 min. Cells were harvested as described above and gently suspended in 0.04 volumes of ice-cold TFB II (see Appendix 1). Aliquots (100 µl) of cells were stored at -70ºC.

Chemically-competent DH5α cells were defrosted on ice and a 10 µl ligation reaction (section 2.4.13) was added to 100 µl of cells. The cell-ligation mixture was kept on ice for 30 min before it was heat-shocked at 42ºC in a waterbath for 45 sec. Immediately after heat-shock, the transformation reaction was placed on ice for 2 min before 350 µl of SOC medium (Invitrogen) was added, and the cells suspended by pipetting. Transformants were recovered after incubating at 37ºC for 1 hr by spreading 250 µl of cell suspension onto appropriate antibiotic plates and incubating at 37ºC overnight.

2.4.14.2 Preparation and transformation of electro-competent *E. coli* cells

All plasmid transformations in wild-type VTEC O157 isolates were performed by electroporation. *E. coli* strains for preparation of electro-competent cells were

cultured in the same way to those for chemically-competent cells. Culture pellets were washed twice by suspending in 0.25 volumes of ice-cold 10% (v/v) glycerol and then centrifuging (4,000 x *g*, 8 min at 4ºC). The cell pellet was suspended in 0.005 volumes of ice-cold 10% glycerol and aliquots (45 µl) of cells were stored at -70ºC.

Electro-competent cells were defrosted on ice and 1 µl of purified plasmid DNA was added to 45 µl of cells. The cell-plasmid mixture was kept on ice for 1 min before electroporating at 2.5 kV in a 2 mm gap electroporation cuvette (Flowgen). SOC medium (1 ml) was added immediately and the cells suspended by gentle pipetting. The cell suspension was transferred to a micro-centrifuge tube and transformants were recovered after incubating at 37ºC for 1 hr by spreading 200 µl of cell suspension onto appropriate antibiotic plates and incubating at 37ºC overnight.

2.5 Indirect immunofluorescence staining

2.5.1 Immunostaining of O26 and O157 antigen on *E. coli* isolates

The serogroup of the *E. coli* O26 and *E. coli* O157 isolates was confirmed by immunostaining with appropriate antisera. Isolates were cultured in LB broth overnight at 37°C and a 100 µl aliquot of culture was fixed in 900 µl of 4% paraformaldehyde (PFA). Aliquots of fixed bacteria were air dried onto multispot slides [C.A. Hendley (Essex) Ltd]. Bacterial samples were incubated with rabbit *E. coli* O26 antisera (Mast Assure) or rabbit *E. coli* O157 antisera (Mast Assure), as appropriate, diluted 1:100 in PBS, for 45 min at room temperature. Following three washes with PBS, the samples were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin secondary antibody (Sigma-Aldrich), diluted 1:500 in PBS, for 45 min at room temperature. The slides were washed a further three times with PBS. Slides were examined by fluorescence microscopy and images were captured with Leica software.

2.5.2 Immunostaining of EspA filaments

E. coli O26 bacteria were grown as described for secreted protein analysis (section 2.8.1) and a 50 µl aliquot of culture (OD_{600} of 0.8) was fixed in 100 µl of 4% PFA.

Aliquots of fixed bacteria were air dried onto multispot slides and incubated with mouse anti-EspA monoclonal antibody (T. Chakraborty, Justus Liebig University, Giessen), prepared at 1:100 in PBS, for 1 hour at room temperature. Following three washes with PBS, the samples were incubated with FITC-conjugated sheep anti-mouse immunoglobulin secondary antibody (Sigma-Aldrich), prepared at 1:500 in PBS, for 1 hour at room temperature and then the slides were washed a further three times with PBS. Slides were examined by microscopy and ten fields for each isolate were selected under phase contrast microscopy using an x100 objective. Phase contrast and fluorescent images were captured of each field with Leica software. For each field, the total number of bacteria and the number of bacteria expressing EspA filaments were counted.

2.5.3 Immunostaining of surface fimbriae

Bacteria were cultured on CFA agar and in CFA and CDMT broths for 24 h at 37°C. Bacterial cultures were fixed in 4% PFA and aliquots of fixed bacteria were air dried onto multispot slides. Sfp fimbriae were labelled by incubating with rabbit anti-SfpA serum (Brunder *et al.*, 2001), diluted 1:20,000 in PBS, and then FITC-conjugated anti-rabbit immunoglobulin secondary antibody (Sigma-Aldrich), diluted 1:500 in PBS, for 1 hour at room temperature. Curli fimbriae were labelled using mouse anti-SEF17 monoclonal antibody (Dr R. La Ragione, VLA Weybridge), diluted 1:100 in PBS, and FITC-conjugated anti-mouse immunoglobulin secondary antibody (Sigma-Aldrich), diluted 1:500 in PBS, incubating for 1 hour at room temperature. Slides were examined by fluorescence microscopy using an x100 objective and images were captured with Leica software.

2.6 Motility assays

Motility was determined using soft-agar plates (see Appendix 1), which were prepared the day before use and left at room temperature overnight. A bacterial colony from an LB agar plate was stabbed into the middle of a soft-agar plate using a sterile inoculating needle. Plates were incubated at 37°C overnight.

2.7 Vero cell cytotoxicity assays

2.7.1 Preparation of culture filtrates

E. coli O26 isolates were cultured in DMEM-HEPES, supplemented with $Fe(NO_3)_3$ to a final concentration of 62 μM (iron-replete medium) and 0.62 μM (low-iron medium). *E. coli* O157 isolates were grown in MEM-HEPES, supplemented with glucose (final concentration of 0.2%) and 0.25 μM $Fe(NO_3)_3$. Bacteria were cultured in appropriate medium with shaking (200 rpm) at 37°C for 24 h. Mitomycin C (0.5 μg/ml) was added when required to cultures at an OD_{600} of 0.3. Bacterial cultures were centrifuged (4,000 x *g*, 20 min at 4°C) and supernatants filter sterilised through low-protein-binding 0.45 μm filters (Millipore). Culture filtrates were diluted in fresh medium to normalise for differences in OD_{600} between cultures.

2.7.2 Vero cell cytotoxicity

Vero cells were seeded into 96-well flat-bottom tissue culture plates (Nunc) at a density of 1 x 10^4 cells/well and incubated at 37ºC in 5% CO_2 for 48 h prior to cytotoxicity assays. For *E. coli* O26 samples, two-fold serial dilutions of the culture filtrates were made in serum-free cell culture medium. For *E. coli* O157 samples, culture filtrates were diluted 1 in 3 in serum-free cell culture medium. Samples (200 μl) were added to the Vero cells and the cytotoxicity assay was performed at 37ºC in 5% CO_2 for 72 h. Untreated controls not exposed to bacterial culture filtrates were included on each plate. Following incubation, detached cells and samples were removed from the wells. The remaining Vero cells were washed with PBS and fixed with 2% formalin in PBS. Quantification of viable Vero cells was determined by staining with crystal violet solution (see Appendix 1) for 1 h. Plates were rinsed with dH_2O and air dried. Crystal violet was released from the cells with 10% acetic acid and absorbance measurements were taken at 590 nm. Vero cell survival was calculated relative to untreated controls not exposed to bacterial culture filtrates. For the *E. coli* O26 samples, the verocytotoxin titre was defined as the reciprocal of the highest dilution of the culture filtrate that caused cytotoxicity in 50% of Vero cells. Vero cell cytotoxicity assays were repeated at least two times for the *E. coli* O26 isolates and three times for the *E. coli* O157 isolates.

2.8 Secreted protein analysis

2.8.1 Protein precipitation

E. coli O26 isolates were cultured in DMEM-HEPES and *E. coli* O157 isolates were grown in MEM-HEPES, supplemented with glucose (final concentration of 0.2%) and 0.25 μM $Fe(NO_3)_3$. Bacteria were cultured in appropriate medium with shaking (200 rpm) at 37°C to an OD_{600} of 0.8. Suspensions were centrifuged (4,000 x *g*, 20 min at 4°C) and the supernatants filter sterilised through low-protein-binding 0.45 μm filters. Bovine serum albumin (BSA; NEB) (4 μg/ml) was added to the supernatants and supernatant proteins were precipitated by the addition of 10% (v/v) trichloroacetic acid (TCA) with overnight incubation at 4°C. The precipitates were harvested by centrifugation (4,000 x *g*, 30 min at 4°C) and the protein pellets air dried and suspended in 1.5 M Tris-HCl (pH 8.8). Protein samples were aliquoted and stored at -20°C. Each isolate was cultured on three separate occasions.

2.8.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Secreted protein samples were separated by SDS-PAGE using either Bio-Rad Mini-PROTEAN II gel apparatus (Bio-Rad) or a Hoefer SE600 Midi gel electrophoresis unit. Protein samples were mixed 1:1 with 2 X Laemmli sample buffer (Sigma-Aldrich) and incubated at 100°C on a heated block for 3 min. Protein samples were loaded in a 5% stacking gel [5% acrylamide-bisacrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 0.1% APS, 0.001% TEMED] and resolved in a 12% resolving gel [12% acrylamide-bisacrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.1% APS, 0.001% TEMED] by electrophoresis. Electrophoresis was performed in Tris-glycine running buffer [25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% SDS] at constant voltage (100 V for mini gels and 150 V for midi gels). Molecular weight markers [low molecular weight range SigmaMarker (Sigma-Aldrich) and full range Rainbow molecular weight marker (Amersham Biosciences)] were used to determine protein sizes.

2.8.3 Colloidal blue staining of SDS-PAGE gels

Proteins separated in polyacrylamide gels were visualised by staining with Colloidal blue (Severn Biotech) overnight. Gels were destained in dH_2O and images captured using a Flowgen MultiImage light cabinet and ChemiImager 4000i v.4.04 software.

2.8.4 Western blotting for EspA

Protein samples were separated by SDS-PAGE and transferred from the polyacrylamide gel onto Hybond ECL nitrocellulose membrane (Amersham Biosciences) using a Trans-Blot electrophoretic transfer cell (Bio-Rad). Briefly, the gel and nitrocellulose membrane were 'sandwiched' together between filter paper and sponge pads, pre-soaked in transfer buffer [20 mM Tris, 154 mM glycine, 20% (v/v) methanol], in a gel holder cassette. The transfer of proteins was performed in transfer buffer at constant voltage (60 V) for 90 min. Nitrocellulose membranes were then blocked with 8% (w/v) Marvel milk powder in PBS at 4°C overnight. Membranes were washed three times with 15 min washes in PBST [PBS containing 0.05% (v/v) Tween 20], and then incubated with mouse anti-EspA monoclonal antibody, prepared at a 1:4000 dilution in PBST, for 1 hour at room temperature on a rocking platform. Excess primary antibody was removed with three 15 min washes in PBST and membranes were then incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin secondary antibody (Dako), prepared at 1:4000 in PBST, for 1 hour at room temperature on a rocking platform. Excess secondary antibody was removed with three 15 min washes in PBST. Membranes were then incubated in ECL solution (see Appendix 1) for 5 min at room temperature on a rocking platform. Chemiluminescence was detected on Hyperfilm ECL chemiluminescence film (Amersham Biosciences) developed in a Protec automatic film processor (Optimax). Images were taken using a Flowgen MultiImage light cabinet and ChemiImager 4000i v.4.04 software.

2.9 Adherence assays to Caco-2 cells

2.9.1 Bacterial culture conditions

E. coli O26 isolates were cultured on CFA agar plates and *E. coli* O157 isolates were cultured on CFA agar plates and in CDMT broth, which have both been used

previously to promote fimbrial expression (Brunder *et al.*, 2001; Evans *et al.*, 1977). Bacteria were cultured at 37ºC for 24 h and were harvested from CFA plates in PBS. Bacterial cultures were diluted to an OD_{600} of 0.5.

2.9.2 Adherence assays

Caco-2 cells were seeded into eight-chamber microscope slides (Becton & Dickinson) at a density of 1 x 10^5 cells/well and incubated at 37ºC in 5% CO_2 for 48 h prior to adherence assays. Bacterial cultures (300 µl) were added to the Caco-2 cells and adherence assays were performed at 37ºC in 5% CO_2 for 2 h. Following incubation, the bacterial suspension was removed and the cells were washed with PBS three times prior to fixation with 4% PFA. Bacteria attached to cells were labelled by indirect immunofluorescence by incubating with rabbit *E. coli* O26 antisera or rabbit *E. coli* O157 antisera as appropriate, diluted 1:100 in PBS, for 45 min at room temperature. Following three washes with PBS, the slides were incubated with Alexa Fluor 568 secondary antibody (Molecular Probes), prepared at 1:1,000 in PBS, for 45 min at room temperature, and then the slides were washed a further three times with PBS. Slides were examined by fluorescence microscopy and the number of bacteria per field was counted for randomly selected fields in each well for at least 20 fields. Images were captured with Leica software. Adherence assays were repeated at least three times for each isolate.

2.9.3 Inhibition adherence assays

Bacteria were cultured on CFA agar and before adding bacteria to Caco-2 cells, aliquots of bacterial cultures were incubated for 30 min at room temperature in the presence or absence of a reagent which should interact with a specific adhesin. 3% (w/v) mannose was used to competitively inhibit type 1 fimbrial binding to receptors containing α-$_D$-mannose. Anti-SfpA serum (diluted 1:400) and anti-curli monoclonal antibody (diluted 1:100) were used to attempt to inhibit adherence due to Sfp fimbriae and curli, respectively. Adherence to Caco-2 cells and subsequent staining, visualisation and enumeration of results were carried out as described in section 2.9.2.

2.10 Biofilm assays

Bacteria were cultured statically overnight at 28°C or 37°C in LB, CFA and CDMT broths. Cultures were diluted 1:100 in fresh medium and 200 µl/well was added to 96-well flat-bottom polystyrene tissue culture plates (Nunc). Plates were incubated at 28°C or 37°C for 24 h and 48 h. Bacterial adherence to microtitre well surfaces was determined by crystal violet staining, as described in section 2.7.2, except that washes were done with PBS. Sterile medium was included as a control, and the absorbance (590 nm) was subtracted from the other values. Biofilm assays were repeated three times for each isolate.

2.11 Yeast cell agglutination

A single colony of the isolate to be tested was inoculated into 5 ml LB broth and incubated statically at 37°C for 24 h. The culture was subcultured into a further 5 ml LB broth and incubated statically for another 24 h. Subculturing was repeated once more. Yeast cell agglutination was carried out by mixing 15 µl bacterial culture with an equal volume of baker's yeast suspension (10 mg/ml) on glass slides and the degree of clumping was assessed. Mannose inhibition of agglutination was confirmed using 3% α-$_D$-mannose in the yeast suspension.

2.12 Measurement of plasmid-based promoter-GFP fusions

To investigate *sfpA*, *csgBAC* and *csgD* promoter activity in different VTEC O157 backgrounds, the appropriate isolates were transformed with promoter-GFP translational fusions (see sections 2.4.13 and 2.4.14.2). Single transformants were cultured on CFA agar and in CDMT broth (both containing 25 µl ml^{-1} chloramphenicol [CAM]) for 24 h at 37°C, and bacteria were harvested from CFA-CAM agar plates in CFA-CAM broth. Total green fluorescent protein (GFP) produced by the population was determined by analysing 100 µl aliquots of culture (OD_{600} of 0.5) in a fluorimeter (FLUOstar Optima). Promoterless plasmid pAJR70 in each strain background acted as a control for background fluorescence and was subtracted from the other values. Promoter activity in each isolate was measured on three separate occasions.

2.13 Statistical analysis

Associations between observation data were tested using the chi-square test or Fisher's exact test if data sets contained a small number of observations. For comparisons of Vtx titres from non-induced VTEC O26 isolates, the median Vtx titre values were $\log_{10}$ transformed and analysed using two-sample *t* tests. For comparisons of Vtx titres without and after induction, the Vtx titres values were $\log_{10}$ transformed and differences assessed with the paired *t* test. For the VTEC O26 Caco-2 adherence assays, bacteria per field counts were $\log_{10}$ transformed and analysed by analysis of variance (ANOVA). Individual differences were investigated using Tukey's test for multiple comparisons, with a family error rate of 1%. The VTEC O157 Vero cell cytotoxicity and biofilm assay data were analysed by ANOVA. For the initial VTEC O157 Caco-2 adherence assays and the inhibition adherence assays, bacteria per field counts were $\log_{10}$ transformed and analysed with mixed models using the REML (restricted maximum likelihood) directive, dropping non-significant terms for fixed effects. A difference between predicted means was considered significant if it was greater than twice the standard error of difference. For graphical representation, predicted means and confidence intervals were back-transformed. Caco-2 adherence assays with the curli deletion mutant and the European SF VTEC O157:NM isolates, and the promoter expression data were analysed using two-sample *t* tests. All statistical analyses were performed using Genstat, 10th edition, and Minitab 15.

2.14 Nucleotide sequence accession numbers

Nucleotide sequences from this research have been deposited in the GenBank database under the accession numbers listed in Table 2.3.

Table 2.3. Details and accession numbers of nucleotide sequences determined in this study.

Isolate	Sequence description	Sequence length (bp)	Accession number
H2687	*vtx*$_2$	1,248	EU526759
H8824	*vtx*$_2$	1,248	EU526760
H8432	*vtx*$_2$	1,248	EU526761
H8489	*vtx*$_2$	1,248	EU526762
H8757	*vtx*$_2$	1,248	EU526763
H8478	*vtx*$_2$	1,248	EU526764
340/97	*csgBA* and *csgDEFG* operons and the *csgB*-to-*csgD* intergenic region	4,544	EU554557
080/01	*csgBA* and *csgDEFG* operons and the *csgB*-to-*csgD* intergenic region	4,544	EU554558
E06/486	*csgBA* and *csgDEFG* operons and the *csgB*-to-*csgD* intergenic region	4,544	EU554559
H8824	*csgBA* and *csgDEFG* operons and the *csgB*-to-*csgD* intergenic region	4,544	EU554560

Section I

Characterisation of *Escherichia coli* O26 isolates

Verocytotoxin-producing *E. coli* (VTEC) serogroup O26 has emerged as the most common non-O157 VTEC serotype associated with human infection in continental Europe and has been responsible for serious clinical disease including HUS (Caprioli *et al.*, 1997; Gerber *et al.*, 2002; Tozzi *et al.*, 2003; Zhang *et al.*, 2000). VTEC O26 has exceeded VTEC O157 as the principal cause of haemorrhagic colitis and HUS in Italy (Tozzi *et al.*, 2003). In contrast, although human infections associated with *E. coli* O26 have been recognised in Scotland and the rest of the United Kingdom (McMaster *et al.*, 2001; SCIEH, 2003; Willshaw *et al.*, 2001), they are uncommon and do not appear to result in such serious disease. Instead, Scotland has relatively high rates of human infection with *E. coli* O157:H7, with many cases associated with exposure to livestock, such as cattle and sheep, which are major reservoirs of this pathogen (Chase-Topping *et al.*, 2008). The observation that VTEC O26 strains are common and widely dispersed in Scottish cattle (Pearce *et al.*, 2006) has raised concern that bovine VTEC O26 strains may emerge as the cause of serious human infections in Scotland.

This study had two main objectives. The first was to characterise *E. coli* O26 isolates from different backgrounds to determine whether there are similarities/differences and the second was to investigate the potential of *E. coli* O26 strains from Scottish cattle to cause human infection. Genotypic and phenotypic characterisation of *E. coli* O26 isolates from human infections of varying severity may identify characteristics which allow these strains to cause more serious disease. To achieve this it was necessary to compare *E. coli* O26 isolates from different geographical origins – those isolated from human infections in continental Europe included ones associated with life-threatening HUS whereas those isolated from patients in Scotland were generally associated with simple diarrhoea. Additionally, comparing *E. coli* O26 isolates from Scottish cattle to those recovered from human infections, may indicate whether strains from the Scottish food-chain represent a threat to human health.

Chapter 3

Genotypic characterisation of *Escherichia coli* O26 isolates

The fact that *E. coli* O26 isolates have been recovered from human infections of different clinical severity prompted the investigation of the genomic 'make-up' and gene repertoire from several clinical isolates, with the aim to identify genotypic characteristics which may allow strains to cause more serious clinical disease. In addition, genotypic analysis of *E. coli* O26 isolates from Scottish cattle may indicate the potential of these bovine isolates to cause human infection. This chapter describes the genotypic characterisation of human and bovine *E. coli* O26 isolates by multilocus sequence typing (MLST) and the presence of known virulence genes.

3.1 *E. coli* O26 isolates

Sixty eight *E. coli* O26 isolates from different backgrounds were collected for this study. The collection contained 35 isolates recovered from cases of human infection with different forms of clinical disease and 33 isolates from Scottish cattle. A full description of the isolates used in this study is detailed in section 2.1.2 and Table 2.1(B) and the information stated was provided by the supplier of the isolates.

3.1.1 Confirmation that the isolates were *E. coli* serogroup O26

To confirm that the isolates included in this research were *E. coli* serogroup O26, PCR to detect the presence of the *wzx* (O-unit flippase) and *wzy* (O-unit polymerase) genes, present in the O26 antigen gene cluster (DebRoy *et al.*, 2004), was used to analyse the isolates and reference *E. coli* strains [Table 2.1(A) and (B)]. Reference *E. coli* O26 strains (413/89-1, ZAP078 and ZAP194) were positive for both the O26 *wzx* and *wzy* genes whereas the non-O26 *E. coli* strains tested (*E. coli* O157:H7 strains NCTC 12900 and EDL933, K-12 MG1655 and EPEC O127:H6 strain E2348/69) were negative for these genes. All isolates contained both the O26 *wzx* and *wzy* genes, with the exception of ZAP1110_S1, ZAP1113_S4 and ZAP1148_I31, which carried neither of these genes. This indicated that while the majority of the isolates were serogroup O26, isolates ZAP1110_S1, ZAP1113_S4 and ZAP1148_I31 were not. Examples of the agarose gels obtained following PCR of the O26 *wzx* and *wzy* genes are shown in Figure 3.1.

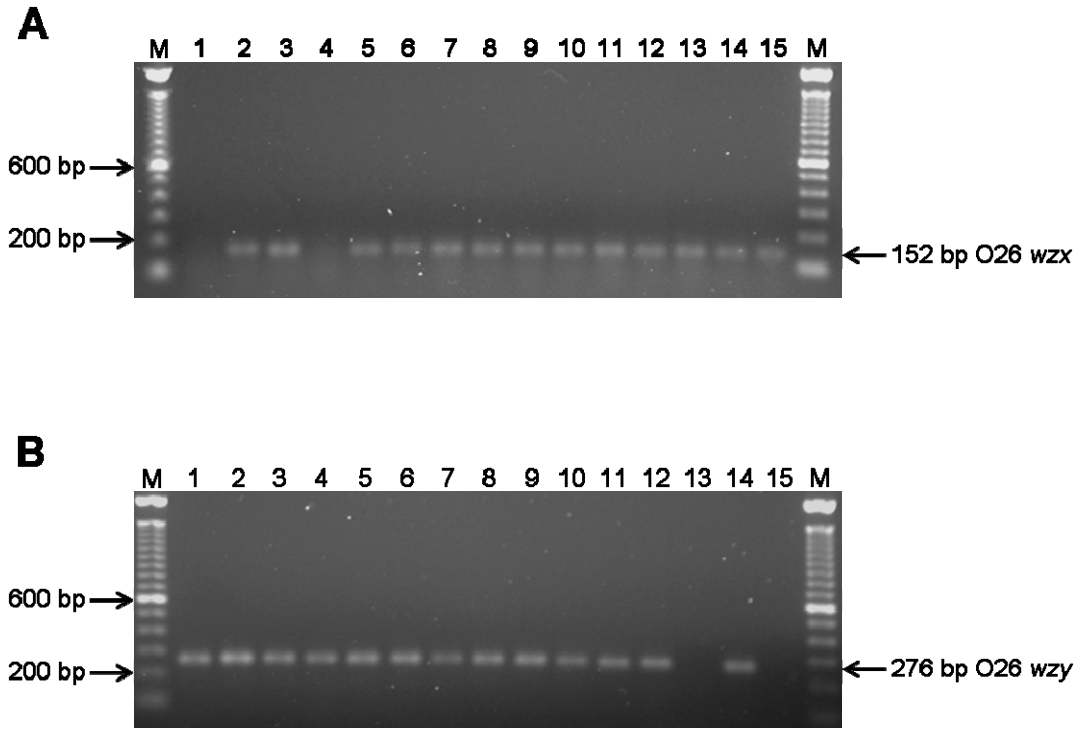


Figure 3.1. PCR detection of O26 *wzx* and *wzy* genes. (A) PCR of O26 *wzx* from isolates ZAP1110_S1 (lane 1), ZAP1111_S2 (lane 2), ZAP1112_S3 (lane 3), ZAP1113_S4 (lane 4), ZAP1114_S6 (lane 5), ZAP1115_S7 (lane 6), ZAP1116_S8 (lane 7), ZAP1117_S9 (lane 8), ZAP1118_S10 (lane 9), ZAP1119_S11 (lane 10), ZAP1120_S12 (lane 11), ZAP1121_G13 (lane 12), ZAP1122_G14 (lane 13), ZAP1123_G15 (lane 14) and ZAP1124_G16 (lane 15). The majority of the isolates generated a 152 bp product indicating the presence of the O26 *wzx* gene. O26 *wzx* was absent in isolates ZAP1110_S1 and ZAP1113_S4. (B) PCR of O26 *wzy* from isolates ZAP1125_G17 (lane 1), ZAP1126_G18 (lane 2), ZAP1127_G19 (lane 3), ZAP1128_G20 (lane 4), ZAP1129_G21 (lane 5), ZAP1130_G22 (lane 6), ZAP1131_G23 (lane 7), ZAP1132_G24 (lane 8), ZAP1133_G25 (lane 9), ZAP1134_G26 (lane 10), ZAP1135_G27 (lane 11) and ZAP1136_G28 (lane 12). These twelve isolates generated a 276 bp product indicating the presence of the O26 *wzy* gene. Lane 13, *E. coli* O157:H7 isolate NCTC 12900 (negative control); lane 14, *E. coli* O26 isolate 413/89-1 (positive control); lane 15, DNA negative (PCR control). Lane M, 100 bp molecular size marker.

Additional confirmation that the isolates were *E. coli* serogroup O26 was provided by immunostaining with O26 antisera. While the majority of the isolates exhibited fluorescent surface staining isolates ZAP1110_S1, ZAP1113_S4 and ZAP1148_I31 were negative. A selection of phase-contrast and fluorescence micrographs is shown in Figure 3.2. Taken together, PCR and immunostaining analysis indicated that all the isolates, with the exception of ZAP1110_S1, ZAP1113_S4 and ZAP1148_I31, were *E. coli* serogroup O26. These isolates were therefore not analysed further in this study.

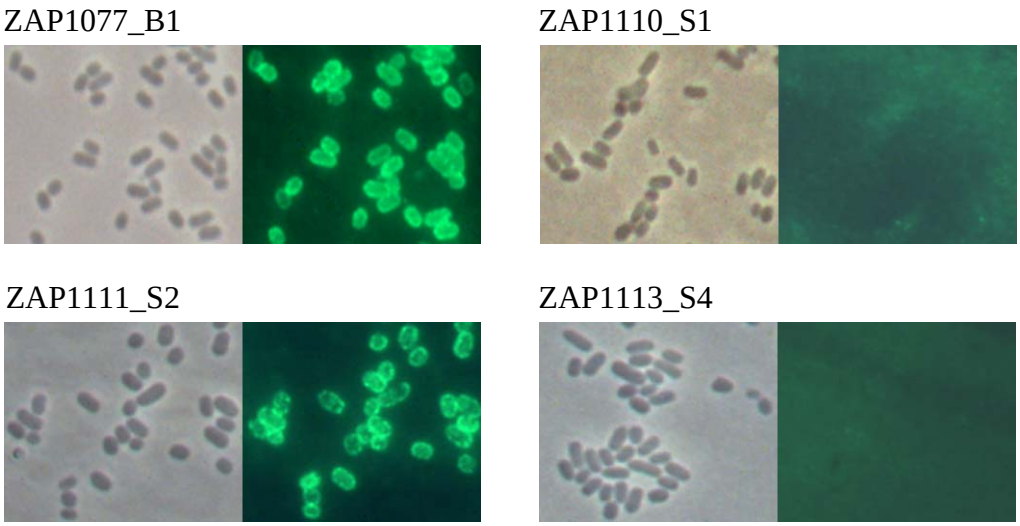


Figure 3.2. Detection of the O26 antigen by immunofluorescence microscopy. Phase-contrast (left panel) and fluorescence (right panel) micrographs are shown for the indicated isolates stained for the O26 antigen using O26 antisera. Slides were examined using an x100 objective and images were captured with Leica software. While the majority of the isolates exhibited fluorescent surface staining, isolates ZAP1110_S1, ZAP1113_S4 and ZAP1148_I31 were negative.

3.2 Multilocus sequence typing (MLST) of *E. coli* O26 isolates

Multilocus sequence typing (MLST) is widely used to investigate the clonal relationship between bacteria (Cooper & Feil, 2004) and it has been used successfully to identify the more virulent clones in certain bacterial species, including *Streptococcus pneumoniae* and *Neisseria meningitidis* (Brueggemann *et al.*, 2003; Maiden *et al.*, 1998). Although pulsed-field gel electrophoresis (PFGE) is the most widely used method for molecular typing of *E. coli* strains, MLST is increasingly being employed as an alternative method (Adiri *et al.*, 2003; Beutin *et al.*, 2005; Gilmour *et al.*, 2005; Wirth *et al.*, 2006). In this study, MLST was performed on 63 *E. coli* O26 isolates from different backgrounds for two main purposes. Firstly, it was used to determine the genetic relatedness among the isolates; to establish if the *E. coli* O26 isolates recovered from patients in Scotland were closely related to those isolated from patients in continental Europe and to ascertain if the bovine isolates were related to the isolates from human infections. Secondly, it permitted an investigation of whether MLST could identify the more virulent *E. coli* O26 pathogens.

3.2.1 Nucleotide sequencing of housekeeping genes

Internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) from the 63 *E. coli* O26 isolates were sequenced and the allele numbers and sequence types (STs) were assigned in accordance with the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli). MLST analysis resolved the 63 *E. coli* O26 isolates into six STs (Table 3.1 and Appendix 2). The allelic profile for each ST is detailed in Table 3.2.

Table 3.1. Multilocus sequence typing of *E. coli* O26 isolates.

Isolate	ST[a]	ST cplx[a]	Isolate	ST[a]	ST cplx[a]
ZAP1077_B1	29	29	ZAP1111_S2	574	29
ZAP1078_B2	21	29	ZAP1112_S3	29	29
ZAP1079_B3	21	29	ZAP1114_S6	21	29
ZAP1080_B4	21	29	ZAP1115_S7	21	29
ZAP1081_B5	21	29	ZAP1116_S8	29	29
ZAP1082_B6	29	29	ZAP1117_S9	10	10
ZAP1083_B7	21	29	ZAP1118_S10	29	29
ZAP1084_B8	21	29	ZAP1119_S11	575	29
ZAP1085_B9	21	29	ZAP1120_S12	29	29
ZAP1086_B10	21	29	ZAP1152_S35	21	29
ZAP1087_B11	986	10	ZAP1153_S36	21	29
ZAP1088_B12	21	29	ZAP1121_G13	29	29
ZAP1089_B13	29	29	ZAP1122_G14	21	29
ZAP1090_B14	29	29	ZAP1124_G16	21	29
ZAP1091_B15	21	29	ZAP1125_G17	29	29
ZAP1092_B16	29	29	ZAP1126_G18	21	29
ZAP1093_B17	21	29	ZAP1127_G19	21	29
ZAP1094_B18	21	29	ZAP1128_G20	29	29
ZAP1095_B19	21	29	ZAP1129_G21	21	29
ZAP1096_B20	29	29	ZAP1130_G22	21	29
ZAP1097_B21	21	29	ZAP1131_G23	21	29
ZAP1098_B22	29	29	ZAP1132_G24	21	29
ZAP1099_B23	29	29	ZAP1133_G25	21	29
ZAP1100_B24	21	29	ZAP1134_G26	29	29
ZAP1101_B25	21	29	ZAP1135_G27	29	29
ZAP1102_B26	29	29	ZAP1146_I29	21	29
ZAP1103_B27	21	29	ZAP1147_I30	21	29
ZAP1104_B28	10	10	ZAP1149_I32	21	29
ZAP1105_B29	21	29	ZAP1150_I33	21	29
ZAP1106_B30	21	29	ZAP1151_I34	21	29
ZAP1107_B31	21	29			
ZAP1108_B32	21	29			
ZAP1109_B33	21	29			

[a] The sequence type (ST) and ST complex were assigned in accordance with the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Table 3.2. The allelic profile for each sequence type (ST) among the *E. coli* O26 isolates.

ST	ST complex	Allelic profile							Number of isolates
		adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	
21	29	16	4	12	16	9	7	7	40
29	29	6	4	12	16	9	7	7	18
574	29	16	135	12	16	9	7	7	1
575	29	6	4	12	116	9	7	7	1
10	10	10	11	4	8	8	8	2	2
986	10	10	134	4	8	8	8	2	1

The most frequently recovered STs among the *E. coli* O26 isolates were ST21 and ST29. Together these two STs accounted for 92% of all of the isolates examined. Strains of ST21 and ST29 differ by a single nucleotide in the *adk* locus (Table 3.2). Due to the close genetic relationship between ST21 and ST29, both these STs were assigned to ST complex 29 (Table 3.2). MLST analysis identified two new STs (ST574 and ST575) which were also assigned to ST complex 29. The ST574 isolate (ZAP1111_S2) differed from strains of ST21 by a single nucleotide in the *fumC* locus (Table 3.2) and the isolate assigned to ST575 (ZAP1119_S11) differed from strains of ST29 by a single nucleotide in the *icd* locus (Table 3.2). Three of the *E. coli* O26 isolates analysed (ZAP1087_B11, ZAP1104_B28, ZAP1117_S9) belonged to a clone (ST complex 10) which is genetically distant (differs at all seven loci) to ST complex 29 (Table 3.2). Two of these isolates were ST10 and one was ST986. ST986 differs from ST10 by a single nucleotide in the *fumC* locus (Table 3.2).

Strains of ST21 and ST29 were isolated from patients in both Scotland and continental Europe and also from cattle (Table 3.1). This indicates that closely related clones of *E. coli* O26 reside in patients from different countries and in different hosts.

The STs of isolates associated with different clinical outcomes was investigated to determine the ability of MLST to identify the more virulent *E. coli* O26 pathogens.

Isolates belonging to both ST21 and ST29 were recovered from patients suffering from disease of varying severity (Table 3.3) and statistical analysis found no evidence of an association between MLST ST and the severity of disease elicited. However, it is interesting to note that the majority (83%) of isolates associated with HUS belonged to ST21 and the majority (67%) of isolates belonging to ST29 were associated with diarrhoea (Table 3.3). This suggests that strains belonging to ST21 may have an increased likelihood of causing serious disease following human infection. The majority (67%) of the Scottish cattle isolates were ST21.

Table 3.3. Sequence types (STs) of *E. coli* O26 isolates associated with different forms of clinical disease.

Clinical form[a]	Number of isolates	Number of isolates that belong to ST				
		10	**21**	**29**	**575**	**986**
Diarrhoea	14	1	6	6	1	0
Bloody Diarrhoea/HC	3	0	2	1	0	0
HUS	12	0	10	2	0	0
Cattle	33	1	22	9	0	1

[a] HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome.

3.2.2 Nucleotide sequencing of *espA* and the promoter for *LEE1*

To supplement the MLST scheme and look for greater genetic discrimination of the *E. coli* O26 isolates, the nucleotide sequence of two additional targets, which may have a higher degree of diversity, was determined. One of the targets was *espA*, which encodes the surface-associated protein, EspA. This gene was selected because it was hypothesised that products exposed on the cell surface are a potential target of the immune system and under possible pressure to mutate. The second target was the region upstream of *LEE1*, which is likely to contain the *LEE1* promoter.

The three isolates which belong to ST complex 10 failed to generate amplicons following PCR of *espA* and the region upstream of *LEE1* and, therefore, could not be included in this analysis. The nucleotide sequence of these two targets was

determined for the remaining 60 isolates. Sequence data at each loci was compared using ClustalX and each unique sequence was given a different allele number; allele numbers were assigned in the order in which they were discovered. The results of this analysis are detailed in Table 3.4 (and Appendix 2).

The nucleotide sequences of *espA* in all but one of the 60 *E. coli* O26 isolates were identical (Table 3.4) and the same as *E. coli* O26 isolate 413/89-1 (GenBank accession no. AJ277443). Isolate ZAP1118_S10 differed from the other isolates by a single nucleotide in *espA* (G at base 19 instead of A) and encoded a protein which differed by a single amino acid residue (Ala at residue 7 instead of Thr).

In contrast to the lack of sequence diversity in *espA*, differences in the nucleotide sequence of the region upstream of *LEE1* were identified among *E. coli* O26 isolates. Six different sequences were discovered for the region upstream of *LEE1* in the 60 *E. coli* O26 isolates examined (Table 3.4) and a comparison of these six alleles is shown in Figure 3.3. Figure 3.3 also displays the nucleotide sequence alignment of these *E. coli* O26 alleles with that from *E. coli* O157:H7, in which LEE regulation has been extensively studied. *E. coli* O26 alleles 1, 2, 3 and 4 only differ from each other by a single nucleotide substitution and, aside from these single nucleotide substitutions, alleles 5 and 6 differ from the other 4 alleles by the insertion of one and two thymine base(s), respectively (Figure 3.3). Interestingly, from the approximately 644 bp sequenced, all sequence variation between the *E. coli* O26 alleles occurs within a 91 bp region upstream of Ler, in the region where regulators are known to act in *E. coli* O157:H7 (Figure 3.3).

Table 3.4. Allele numbers for *espA* and the region upstream of *LEE1* from *E. coli* O26 isolates.

Isolate	ST[a]	Allele number[b]	
		espA	upstream of *LEE1*
ZAP1077_B1	29	1	3
ZAP1078_B2	21	1	1
ZAP1079_B3	21	1	2
ZAP1080_B4	21	1	2
ZAP1081_B5	21	1	2
ZAP1082_B6	29	1	1
ZAP1083_B7	21	1	2
ZAP1084_B8	21	1	2
ZAP1085_B9	21	1	2
ZAP1086_B10	21	1	2
ZAP1087_B11	986	ND	ND
ZAP1088_B12	21	1	2
ZAP1089_B13	29	1	4
ZAP1090_B14	29	1	1
ZAP1091_B15	21	1	2
ZAP1092_B16	29	1	4
ZAP1093_B17	21	1	2
ZAP1094_B18	21	1	2
ZAP1095_B19	21	1	2
ZAP1096_B20	29	1	1
ZAP1097_B21	21	1	2
ZAP1098_B22	29	1	4
ZAP1099_B23	29	1	1
ZAP1100_B24	21	1	2
ZAP1101_B25	21	1	2
ZAP1102_B26	29	1	1
ZAP1103_B27	21	1	2
ZAP1104_B28	10	ND	ND
ZAP1105_B29	21	1	1
ZAP1106_B30	21	1	2
ZAP1107_B31	21	1	2
ZAP1108_B32	21	1	2
ZAP1109_B33	21	1	2
ZAP1111_S2	574	1	2
ZAP1112_S3	29	1	4
ZAP1114_S6	21	1	2
ZAP1115_S7	21	1	2
ZAP1116_S8	29	1	1
ZAP1117_S9	10	ND	ND
ZAP1118_S10	29	2	5
ZAP1119_S11	575	1	1
ZAP1120_S12	29	1	6
ZAP1152_S35	21	1	2
ZAP1153_S36	21	1	2
ZAP1121_G13	29	1	1
ZAP1122_G14	21	1	2
ZAP1124_G16	21	1	2
ZAP1125_G17	29	1	1
ZAP1126_G18	21	1	1
ZAP1127_G19	21	1	2
ZAP1128_G20	29	1	1
ZAP1129_G21	21	1	2
ZAP1130_G22	21	1	1
ZAP1131_G23	21	1	1
ZAP1132_G24	21	1	2
ZAP1133_G25	21	1	1
ZAP1134_G26	29	1	1
ZAP1135_G27	29	1	1
ZAP1146_I29	21	1	1
ZAP1147_I30	21	1	1
ZAP1149_I32	21	1	1
ZAP1150_I33	21	1	1
ZAP1151_I34	21	1	1

[a] The sequence type (ST) was assigned in accordance with the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli) following sequencing of housekeeping genes.
[b] The allele numbers at each loci were assigned in the order in which they were discovered; ND, not determined.

Figure 3.3.

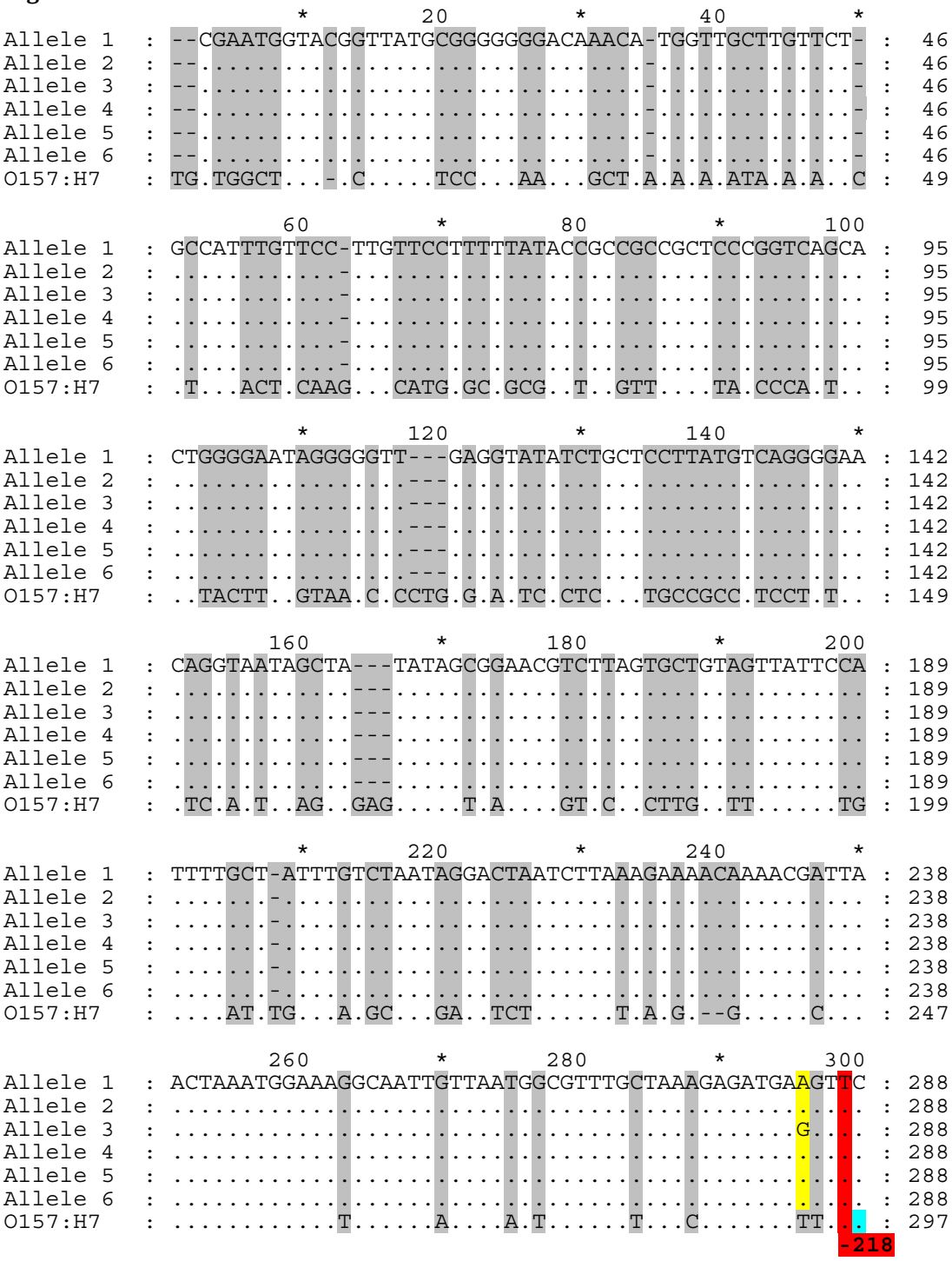
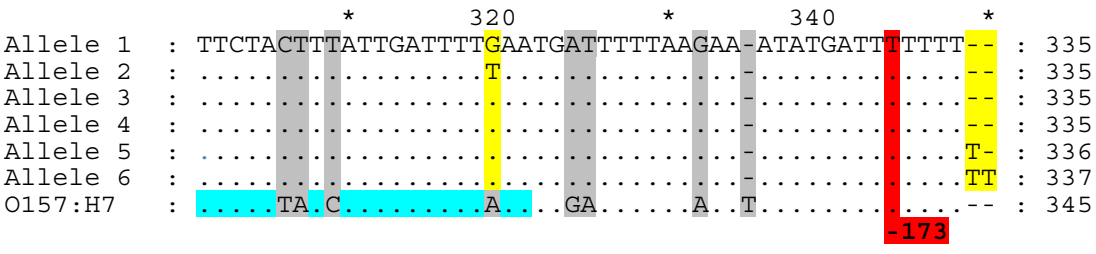


Figure 3.3 ***continued***



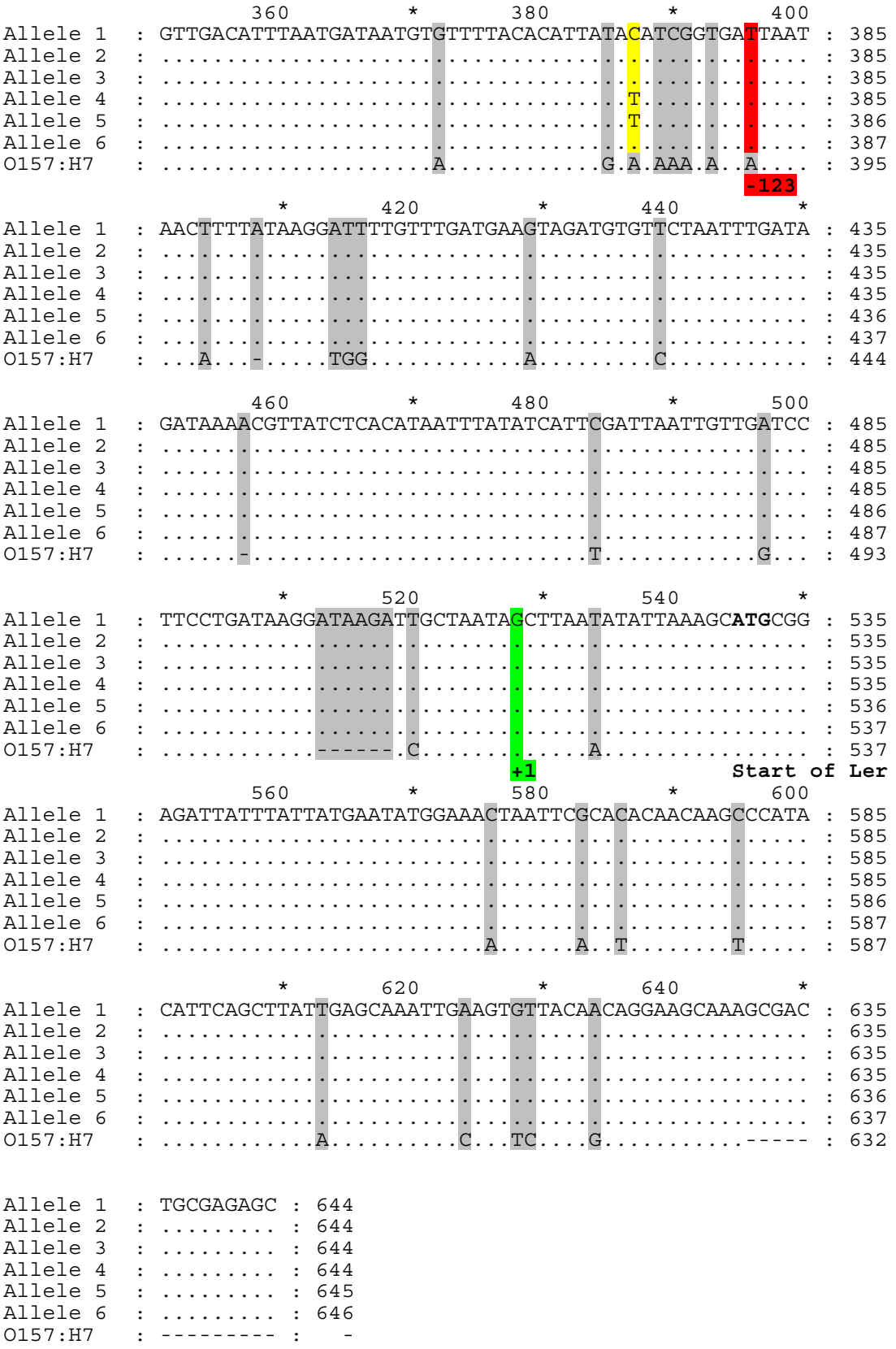


Figure 3.3. Alignment of the nucleotide sequences of the six alleles discovered for the region upstream of *LEE1* in *E. coli* O26 isolates and the region upstream of *LEE1* in *E.*

***coli* O157:H7 TUV93-0.** Approximately 644 bp of sequence data was determined and the numbering on the right-hand side reflects this. Dots indicate nucleotides identical to those of the top sequence (allele 1 of *E. coli* O26) and hyphens (-) indicate the absence of nucleotides. Yellow shaded letters highlight nucleotide differences between the *E. coli* O26 alleles. Grey shaded areas denote nucleotide differences between *E. coli* O26 and *E. coli* O157:H7. Red shaded nucleotides indicate the general areas of the *LEE1* promoter region where regulators act in *E. coli* O157:H7 [information obtained from Sharp & Sperandio (2007); see Appendix 3] and the numbering of these nucleotides is in relation to the P2 *LEE1* promoter transcriptional start site (marked +1 and highlighted in green). Turquoise shaded area corresponds to the IHF (integration host factor)-binding site in *E. coli* O157:H7 (information obtained from Sharp & Sperandio, 2007). The start codon of Ler is marked by nucleotides in boldface type.

From the six alleles discovered among the *E. coli* O26 isolates, the two most prevalent were alleles 1 and 2 which were present in 23/60 (38%) and 30/60 (50%) of the isolates, respectively. The sequence of the region upstream of *LEE1* possessed by *E. coli* O26 isolates belonging to different MLST STs is detailed in Table 3.5. Allele 2 was only ever discovered in isolates belonging to ST21 or the closely related ST574 and statistical analysis found strong evidence of an association ($p < 0.001$) between the carriage of allele 2 by isolates and them belonging to ST21. Allele 1, however, was present in equal numbers of ST21 and ST29 isolates and in the isolate belonging to ST575. The less common alleles, alleles 3, 4, 5 and 6, were only detected in isolates belonging to ST29.

Table 3.5. Prevalence of alleles for the region upstream of *LEE1* among *E. coli* O26 isolates belonging to different MLST STs.

ST	Number of isolates	Number of isolates possessing allele number for region upstream of *LEE1*					
		1	**2**	**3**	**4**	**5**	**6**
21	40	11	29	0	0	0	0
29	18	11	0	1	4	1	1
574	1	0	1	0	0	0	0
575	1	1	0	0	0	0	0

The sequence of the region upstream of *LEE1* possessed by *E. coli* O26 isolates recovered from different backgrounds is detailed in Table 3.6. There is strong evidence of an association ($p = 0.001$) between the origin of the isolate and the sequence of the region upstream of *LEE1*. The vast majority (14/19, 74%) of human isolates recovered in continental Europe carried allele 1 whereas only a limited number of isolates from patients and cattle in Scotland possessed this allele (20% and 23%, respectively) (Table 3.6). Instead, half of the human isolates recovered in Scotland and the majority of Scottish cattle isolates harboured allele 2 (Table 3.6).

Table 3.6. Prevalence of alleles for the region upstream of *LEE1* among *E. coli* O26 isolates recovered from different backgrounds.

Origin of isolate	Number of isolates	Number of isolates possessing allele number for region upstream of *LEE1*					
		1	**2**	**3**	**4**	**5**	**6**
Human (Scotland)	10	2	5	0	1	1	1
Human (continental Europe)	19	14	5	0	0	0	0
Cattle	31	7	20	1	3	0	0

There is no evidence of an association between the sequence of the region upstream of *LEE1* and the severity of disease following human infection. For both allele 1 and allele 2, a similar or equivalent number of isolates were associated with both simple diarrhoea and HUS.

3.2.3 Summary of MLST results

Nucleotide sequencing of housekeeping genes found very little genetic variation in the genomic 'backbone' among the vast majority of *E. coli* O26 isolates indicating that closely related strains reside in patients from different countries and in different hosts. Only three isolates had marked variation in their genomic 'backbone' and were genetically distant from the other isolates. Nucleotide sequencing of the region upstream of *LEE1* found sequence differences between isolates recovered from different backgrounds. While the majority of human isolates recovered in continental Europe carried allele 1, most of the isolates from patients and cattle in Scotland harboured allele 2.

3.3 Virulence gene carriage by *E. coli* O26 isolates

Further genotypic characterisation of the *E. coli* O26 isolates was achieved by determining their carriage of known virulence factors. All isolates were screened by PCR for the genes encoding verocytotoxin 1 (vtx_1), verocytotoxin 2 (vtx_2) and enterohaemolysin (*hlyA*). The possession of the LEE pathogenicity island was determined by screening for intimin (*eae*) and SepL (*sepL*) by PCR. Examples of the agarose gels obtained following multiplex PCR of vtx_1, vtx_2, *eae* and *hlyA* genes and PCR of *sepL* are shown in Figure 3.4. The virulence genes carried by each of the *E. coli* O26 isolates is detailed in Table 3.7 (and Appendix 2).

The virulence gene profile for isolates ZAP1123_G15 and ZAP1136_G28 did not agree with the information provided by the supplier of the isolates. ZAP1123_G15 was recorded as positive for vtx_2 but was found to be positive for vtx_1 and ZAP1136_G28 was shown to be positive for vtx_2 although it should have been positive for vtx_1. Due to the uncertainty with regard to the information about these two isolates, ZAP1123_G15 and ZAP1136_G28 were omitted from further study.

The major virulence factor of *E. coli* strains is the production of verocytotoxin (Vtx). More than half (55%) of the *E. coli* O26 isolates recovered from patients in Scotland did not possess a gene encoding Vtx. Of the five isolates which were *vtx* gene positive, three carried vtx_1 alone and two possessed vtx_1 in combination with vtx_2. No Scottish human isolate carried the vtx_2 gene alone. All combinations of *vtx* gene carriage were observed among the *E. coli* O26 isolates recovered from patients in continental Europe; five carried vtx_1 alone, six carried vtx_2 alone, four carried vtx_1 and vtx_2 and four were *vtx* gene negative. Among the 33 Scottish cattle isolates analysed 23 harboured *vtx* genes - sixteen possessed vtx_1 alone and seven possessed both vtx_1 and vtx_2. No *E. coli* O26 isolated from Scottish cattle carried the vtx_2 gene alone.

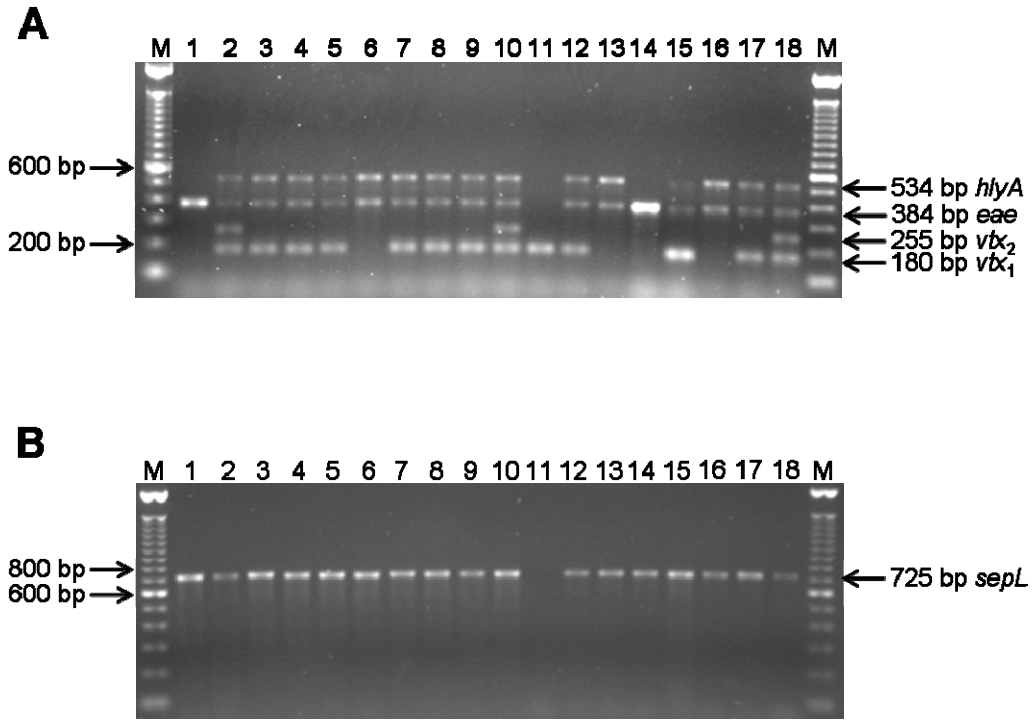


Figure 3.4. PCR detection of virulence genes. (A) Multiplex PCR of vtx_1, vtx_2, *eae* and *hlyA* genes and (B) PCR of *sepL* from isolates ZAP1077_B1 (lane 1), ZAP1078_B2 (lane 2), ZAP1079_B3 (lane 3), ZAP1080_B4 (lane 4), ZAP1081_B5 (lane 5), ZAP1082_B6 (lane 6), ZAP1083_B7 (lane 7), ZAP1084_B8 (lane 8), ZAP1085_B9 (lane 9), ZAP1086_B10 (lane 10), ZAP1087_B11 (lane 11), ZAP1088_B12 (lane 12), ZAP1089_B13 (lane 13), ZAP1090_B14 (lane 14), ZAP1091_B15 (lane 15), ZAP1092_B16 (lane 16), ZAP1093_B17 (lane 17) and ZAP1094_B18 (lane 18). Lane M, 100 bp molecular size marker.

Table 3.7. PCR detection of virulence genes carried by *E. coli* O26 isolates[a].

Isolate	*vtx*[b]	LEE		*hlyA*
		eae	*sepL*	
ZAP1077_B1	–	+	+	–
ZAP1078_B2	1, 2	+	+	+
ZAP1079_B3	1	+	+	+
ZAP1080_B4	1	+	+	+
ZAP1081_B5	1	+	+	+
ZAP1082_B6	–	+	+	+
ZAP1083_B7	1	+	+	+
ZAP1084_B8	1	+	+	+
ZAP1085_B9	1	+	+	+
ZAP1086_B10	1, 2	+	+	+
ZAP1087_B11	1	–	–	–
ZAP1088_B12	1	+	+	+
ZAP1089_B13	–	+	+	+
ZAP1090_B14	–	+	+	–
ZAP1091_B15	1	+	+	+
ZAP1092_B16	–	+	+	+
ZAP1093_B17	1	+	+	+
ZAP1094_B18	1, 2	+	+	+
ZAP1095_B19	1	+	+	+
ZAP1096_B20	–	+	+	+
ZAP1097_B21	1	+	+	+
ZAP1098_B22	–	+	+	+
ZAP1099_B23	–	+	+	–
ZAP1100_B24	1, 2	+	+	+
ZAP1101_B25	1	+	+	+
ZAP1102_B26	–	+	+	–
ZAP1103_B27	1	+	+	+
ZAP1104_B28	–	–	–	–
ZAP1105_B29	1, 2	+	+	+
ZAP1106_B30	1, 2	+	+	+
ZAP1107_B31	1	+	+	+
ZAP1108_B32	1	+	+	+
ZAP1109_B33	1, 2	+	+	+
ZAP1111_S2	1	+	+	+
ZAP1112_S3	–	+	+	+
ZAP1114_S6	1	+	+	+
ZAP1115_S7	1	+	+	+
ZAP1116_S8	–	+	+	–
ZAP1117_S9	–	–	–	–
ZAP1118_S10	–	+	+	+
ZAP1119_S11	–	+	+	+
ZAP1120_S12	–	+	+	–
ZAP1152_S35	1, 2	+	+	+
ZAP1153_S36	1, 2	+	+	+

Table 3.7 ***continued***

Isolate	*vtx*[b]	LEE		*hlyA*
		eae	*sepL*	
ZAP1121_G13	–	+	+	–
ZAP1122_G14	1, 2	+	+	+
ZAP1123_G15	1	+	+	–
ZAP1124_G16	1	+	+	–
ZAP1125_G17	–	+	+	+
ZAP1126_G18	1, 2	+	+	–
ZAP1127_G19	1	+	+	+
ZAP1128_G20	2	+	+	+
ZAP1129_G21	1, 2	+	+	–
ZAP1130_G22	1, 2	+	+	+
ZAP1131_G23	1	+	+	–
ZAP1132_G24	2	+	+	–
ZAP1133_G25	–	+	+	–
ZAP1134_G26	2	+	+	+
ZAP1135_G27	–	+	+	+
ZAP1136_G28	2	+	+	+
ZAP1146_I29	1	+	+	+
ZAP1147_I30	2	+	+	+
ZAP1149_I32	2	+	+	+
ZAP1150_I33	2	+	+	+
ZAP1151_I34	1	+	+	+

[a] +, gene present; –, gene absent.
[b] *vtx* types: 1, vtx_1; 2, vtx_2; 1, 2, vtx_1 and vtx_2; –, *vtx* negative.

The vast majority (95%) of the *E. coli* O26 isolates examined carried the LEE pathogenicity island. Only three isolates did not harbour the LEE; one was isolated from a patient in Scotland and two were isolated from Scottish cattle. To determine if the LEE is inserted in the *pheU* tRNA gene, as in *E. coli* O26 isolate 413/89-1, PCRs were performed on all LEE-positive isolates using primer pairs that link the 5' and 3' ends of the island with the adjacent region of *pheU*. Primer pair cad1-LS1 (Table 2.2) amplifies the left junction of the pathogenicity island inserted into *pheU*, producing a 947 bp amplicon, and primer pair espB73-espB52 (Table 2.2) amplifies the right junction of the island inserted into *pheU*, yielding a 1.48 kb product. Amplicons of the expected size were obtained in both of these PCRs for the majority of the *E. coli* O26 isolates examined. However, for some of the isolates, only one junction of the LEE inserted in *pheU* was positive by PCR. This may be explained by variation in the DNA sequence at the ends of the LEE between isolates. This analysis demonstrated that in LEE-positive *E. coli* O26 isolates the LEE pathogenicity island is always inserted in *pheU*. The insertion of LEE in *pheU* was confirmed in each of these isolates by the absence of a product in the PCR, using primer pair cad1-espB52, designed to span intact *pheU*.

Forty seven (75%) of the 63 *E. coli* O26 isolates analysed possessed the *hlyA* gene. Of the LEE-positive VTEC O26 isolates, 100% of Scottish human isolates, 67% of European human isolates and 100% of Scottish cattle isolates carried *hlyA*.

The carriage of virulence genes by *E. coli* O26 isolated from patients suffering from different forms of clinical disease is shown in Table 3.8. The majority (9/14, 64%) of the isolates associated with diarrhoea were *vtx*-negative. Of the five VTEC O26 isolates associated with diarrhoea, four possessed the vtx_1 gene alone and only one isolate carried both vtx_1 and vtx_2. No isolate associated with diarrhoea harboured the vtx_2 gene alone. The three isolates associated with bloody diarrhoea or haemorrhagic colitis had different Vtx genotypes. While one isolate carried vtx_2 alone and one possessed both vtx_1 and vtx_2, the third isolate was *vtx*-negative. Every *E. coli* O26 isolate associated with HUS was *vtx*-positive and harboured either vtx_1, vtx_2 or vtx_1 in combination with vtx_2. The LEE was detected in 93% of isolates associated with

diarrhoea and 100% of isolates associated with bloody diarrhoea, haemorrhagic colitis and HUS. The *hlyA* gene was present in 9/14 isolates associated with diarrhoea, 2/3 isolates associated with bloody diarrhoea or haemorrhagic colitis and 8/12 isolates associated with HUS.

Table 3.8. Prevalence of virulence genes among *E. coli* O26 isolates associated with different forms of clinical disease.

Clinical form[a]	Number of isolates	Number of isolates positive					
		vtx$_1$	*vtx*$_2$	*vtx*$_1$*vtx*$_2$	*vtx* neg	LEE	*hly*
Diarrhoea	14	4	0	1	9	13	9
BD/HC	3	0	1	1	1	3	2
HUS	12	3	5	4	0	12	8
Cattle	33	16	0	7	10	31	27

[a] BD, bloody diarrhoea; HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome.

The virulence genes carried by *E. coli* O26 isolates belonging to different MLST STs is detailed in Table 3.9. All but one of the 40 ST21 isolates analysed were *vtx*-positive and the closely related ST574 strain harboured *vtx*$_1$. The vast majority (16/18, 89%) of the ST29 isolates did not possess a *vtx* gene; the two exceptions to this were isolates recovered from patients in Germany which contained the *vtx*$_2$ gene. The ST575 isolate which was most closely related to ST29 strains was also *vtx*-negative. The three *E. coli* O26 isolates which belonged to ST complex 10 were the only strains analysed which did not contain the LEE pathogenicity island. While the two ST10 isolates were *vtx*-negative the ST986 isolate carried the *vtx*$_1$ gene.

Table 3.9. Prevalence of virulence genes among *E. coli* O26 isolates belonging to different MLST STs.

ST	ST complex	No. of isolates	Number of isolates positive					
			vtx$_1$	*vtx*$_2$	*vtx*$_1$*vtx*$_2$	*vtx* neg	LEE	*hly*
21	29	40	22	4	13	1	40	34
29	29	18	0	2	0	16	18	11
574	29	1	1	0	0	0	1	1
575	29	1	0	0	0	1	1	1
10	10	2	0	0	0	2	0	0
986	10	1	1	0	0	0	0	0

The carriage of *vtx* genes by *E. coli* O26 isolates possessing different sequences for the region upstream of *LEE1* is shown in Table 3.10. Statistical analysis found strong evidence of an association ($p < 0.001$) between the carriage of *vtx* genes and the nucleotide sequence for the region upstream of *LEE1*. Although similar numbers of isolates carrying allele 1 for the region upstream of *LEE1* were *vtx*-positive and *vtx*-negative (12/23 and 11/23, respectively), allele 2 was only discovered in *vtx*-positive isolates and the less common alleles (alleles 3, 4, 5 and 6) were only detected in *vtx*-negative isolates.

It is interesting to note that while all (5/5) of the *vtx*-positive Scottish human isolates and the vast majority (20/22, 91%) of the *vtx*-positive Scottish cattle isolates carried allele 2, only a limited number (5/15, 33%) of the *vtx*-positive isolates from patients in continental Europe harboured this allele (Table 3.10). The majority (10/15, 67%) of *vtx*-positive European human isolates carried allele 1 (Table 3.10).

Table 3.10. Prevalence of verocytotoxin genes among *E. coli* O26 isolates possessing different sequences for the region upstream of *LEE1*.

Allele number for region upstream of *LEE1*, origin of isolate	Number of isolates	Number of isolates positive			
		vtx$_1$	*vtx*$_2$	*vtx*$_1$*vtx*$_2$	*vtx* neg
Allele 1	23	3	5	4	11
Human (Scotland)	2	0	0	0	2
Human (cont. Europe)	14	3	5	2	4
Cattle	7	0	0	2	5
Allele 2	30	20	1	9	0
Human (Scotland)	5	3	0	2	0
Human (cont. Europe)	5	2	1	2	0
Cattle	20	15	0	5	0
Allele 3	1	0	0	0	1
Cattle	1	0	0	0	1
Allele 4	4	0	0	0	4
Human (Scotland)	1	0	0	0	1
Cattle	3	0	0	0	3
Allele 5	1	0	0	0	1
Human (Scotland)	1	0	0	0	1
Allele 6	1	0	0	0	1
Human (Scotland)	1	0	0	0	1

3.3.1 Summary of virulence gene carriage results

The majority of *E. coli* O26 isolates recovered from patients in Scotland were *vtx*-negative but among those which were *vtx*-positive, only the carriage of *vtx*$_1$ alone or *vtx*$_1$ in combination with *vtx*$_2$ was detected. No Scottish human isolate carried the *vtx*$_2$ gene alone. Among the isolates recovered from patients in continental Europe, all combinations of *vtx* gene carriage were observed (*vtx*$_1$ alone, *vtx*$_2$ alone, *vtx*$_1$ and *vtx*$_2$ and *vtx*-negative). Among the isolates recovered from Scottish cattle the presence of the *vtx*$_1$ gene alone and *vtx*$_1$ with *vtx*$_2$ was detected. No Scottish cattle isolate carried *vtx*$_2$ alone. The vast majority of *E. coli* O26 isolates carried the LEE pathogenicity island, which is always inserted in the *pheU* tRNA gene.

Although the majority of isolates associated with diarrhoea were *vtx* gene negative, isolates which carried either vtx_1 alone or both vtx_1 and vtx_2 were also detected. No isolate associated with diarrhoea possessed vtx_2 alone. In contrast, every isolate associated with HUS was *vtx*-positive and harboured either vtx_1 alone, vtx_2 alone or vtx_1 in combination with vtx_2.

In general, *E. coli* O26 isolates that belonged to MLST ST21 were *vtx* gene positive and those that belonged to ST29 were *vtx*-negative. *E. coli* O26 that did not contain the LEE were genetically distant to the LEE-positive isolates. Isolates which carried allele 1 for their sequence of the region upstream of *LEE1* were either *vtx*-positive or *vtx*-negative whereas those which carried allele 2 were *vtx*-positive and those which carried alleles 3, 4, 5 or 6 were *vtx*-negative. The majority of *vtx*-positive isolates recovered in Scotland, from both human and bovine hosts, carried allele 2 for their sequence of the region upstream of *LEE1* whereas the majority of *vtx*-positive isolates recovered from humans in continental Europe harboured allele 1.

3.4 Carriage of *tccP* and *tccP2* by *E. coli* O26 isolates

The ability of *E. coli* strains to colonise the intestinal mucosa via the formation of attaching and effacing (A/E) lesions is important for their pathogenicity. A characteristic feature of the A/E lesion is the formation of an actin-rich pedestal-like structure in the host cell underneath intimately attached bacteria (Kaper *et al.*, 1998). Until recently, it was considered that EPEC and EHEC induce actin polymerisation by distinct mechanisms. A detailed description of the actin polymerisation pathways in EPEC and EHEC is given in section 1.7.2.2.1. Briefly, actin polymerisation by EPEC O127:H6 requires tyrosine (Y474) phosphorylation of Tir and subsequent recruitment of the N-WASP activator protein, Nck, whereas actin polymerisation by EHEC O157:H7 utilises the N-WASP activator TccP. The gene *tccP* is carried on prophage CP-933U/Sp14 and TccP consists of a unique 80 amino acid amino-terminal region and several almost identical 47 amino acid proline-rich repeats (PRRs) (Campellone *et al.*, 2004; Garmendia *et al.*, 2006). TccP2 is functionally equivalent to TccP and the gene which encodes it (*tccP2*) has been found in SF EHEC O157:NM and the majority of non-O157 EHEC (serogroups O26, O103, O111, O121 and O145) (Ogura *et al.*, 2007).

The importance of TccP and TccP2 in actin polymerisation and subsequent A/E lesion formation prompted an investigation of the carriage of *tccP* and *tccP2* in *E. coli* O26 isolates, with the aim to determine whether the possession of these genes was associated with the ability of *E. coli* O26 to cause more serious clinical disease. In addition, it also allowed an investigation of whether there was a difference in the distribution of *tccP* and *tccP2* between cattle and human isolates.

The presence of *tccP* and *tccP2* genes in the 63 *E. coli* O26 isolates was determined by PCR. The detection of both *tccP* and *tccP2* gene sequences was achieved using a universal primer pair. Universal primers found that the majority (62%) of the isolates analysed were positive for *tccP*/*tccP2*. Single amplicons, which varied in size between isolates, were observed for all *tccP*/*tccP2*-positive isolates, with the exception of ZAP1125_G17, which yielded two distinct fragments.

The specific detection of *tccP* and *tccP2* was achieved using gene-specific primers. All isolates which were positive for universal *tccP*/*tccP2* were found to be negative for *tccP* and positive for *tccP2*. The *tccP2* amplicons exhibited variation in size, ranging from approximately 750 bp to 1,400 bp (Table 3.11). On a few occasions, two products were observed following PCR of *tccP2*. Generally, there was always a prominent band and a second, much fainter, band. The fainter band was probably the consequence of non-specific primer annealing and so only the size of the prominent band was recorded. Isolate ZAP1125_G17 was the exception to this in that *tccP2* PCR generated two well-defined products of equal intensities. It is likely that ZAP1125_G17 possesses two copies of *tccP2* with different numbers of PRRs. It should be noted that due to sequence variation, the primers used in this screen might not amplify all the *tccP*/*tccP2* variants.

The three isolates that were LEE-negative (ZAP1087_B11, ZAP1104_B28 and ZAP1117_S9) did not carry the *tccP2* gene (Table 3.11) and were omitted from further analysis in this section. Analysis of the sixty LEE-positive *E. coli* O26 found that while only half (5/10) of the isolates recovered from patients in Scotland possessed the gene encoding TccP2, the vast majority (17/19, 89%) of isolates recovered from patients in continental Europe harboured this gene. Among the 31 LEE-positive Scottish cattle isolates analysed, only 17 (55%) carried the *tccP2* gene (Table 3.11). Analysis of *tccP2* amplicon size in relation to the origin of the isolate found that *tccP2*-positive *E. coli* O26 isolated in continental Europe possessed only the smaller variants of *tccP2* (750 bp, 850 bp and 900 bp) (Table 3.11). None of the *tccP2*-positive Scottish clinical isolates harboured the 750 bp or 850 bp *tccP2* variants; they generally carried a larger *tccP2* gene (ranging from 900 bp to 1,400 bp) (Table 3.11). *tccP2* amplicon sizes from the Scottish cattle isolates were comparable to both groups of human isolates (ranging from 750 bp to 1,050 bp) (Table 3.11).

Table 3.11. PCR detection of *tccP2* among *E. coli* O26 isolates.

Isolate	*tccP2*[a]	Isolate	*tccP2*[a]
ZAP1077_B1	+ (1050bp)	ZAP1111_S2	–
ZAP1078_B2	–	ZAP1112_S3	+ (900bp)
ZAP1079_B3	–	ZAP1114_S6	–
ZAP1080_B4	+ (750bp)	ZAP1115_S7	–
ZAP1081_B5	+ (750bp)	ZAP1116_S8	+ (1050bp)
ZAP1082_B6	+ (1050bp)	ZAP1117_S9	–
ZAP1083_B7	–	ZAP1118_S10	+ (950bp)
ZAP1084_B8	–	ZAP1119_S11	+ (1250bp)
ZAP1085_B9	–	ZAP1120_S12	+ (1400bp)
ZAP1086_B10	–	ZAP1152_S35	–
ZAP1087_B11	–	ZAP1153_S36	–
ZAP1088_B12	–	ZAP1121_G13	+ (900bp)
ZAP1089_B13	+ (750bp)	ZAP1122_G14	+ (750bp)
ZAP1090_B14	+ (1050bp)	ZAP1124_G16	+ (750bp)
ZAP1091_B15	+ (750bp)	ZAP1125_G17	+ (900bp, 750bp)
ZAP1092_B16	+ (900bp)	ZAP1126_G18	+ (750bp)
ZAP1093_B17	–	ZAP1127_G19	+ (750bp)
ZAP1094_B18	–	ZAP1128_G20	+ (850bp)
ZAP1095_B19	+ (750bp)	ZAP1129_G21	+ (750bp)
ZAP1096_B20	+ (1050bp)	ZAP1130_G22	+ (750bp)
ZAP1097_B21	–	ZAP1131_G23	+ (750bp)
ZAP1098_B22	+ (750bp)	ZAP1132_G24	–
ZAP1099_B23	+ (1050bp)	ZAP1133_G25	+ (900bp)
ZAP1100_B24	–	ZAP1134_G26	+ (900bp)
ZAP1101_B25	+ (750bp)	ZAP1135_G27	+ (900bp)
ZAP1102_B26	+ (1050bp)	ZAP1146_I29	–
ZAP1103_B27	+ (750bp)	ZAP1147_I30	+ (900bp)
ZAP1104_B28	–	ZAP1149_I32	+ (900bp)
ZAP1105_B29	+ (750bp)	ZAP1150_I33	+ (900bp)
ZAP1106_B30	–	ZAP1151_I34	+ (750bp)
ZAP1107_B31	–		
ZAP1108_B32	+ (750bp)		
ZAP1109_B33	–		

[a] +, gene present (PCR fragment size(s) are indicated in parentheses); –, gene absent.

The carriage of *tccP2* by *E. coli* O26 isolated from patients suffering from different forms of clinical disease is shown in Table 3.12. There was no evidence of an association between the possession of *tccP2* and the ability of *E. coli* O26 to cause more serious clinical disease. The majority (22/28, 79%) of clinical isolates carried *tccP2*, regardless of the severity of disease elicited (Table 3.12).

Table 3.12. Prevalence of *tccP2* among *E. coli* O26 isolates associated with different forms of clinical disease.

Clinical form[a]	Number of isolates	Number of isolates positive for *tccP2*
Diarrhoea	13	10
Bloody Diarrhoea/HC	3	2
HUS	12	10
Cattle	31	17

[a] HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome.

The carriage of *tccP2* by isolates with different Vtx genotypes recovered from different origins is detailed in Table 3.13. Notably, 100% of the *vtx*-negative isolates, regardless of country of origin or host, carried the *tccP2* gene (Table 3.13). Interestingly, while none of the five VTEC O26 isolates recovered from clinical cases in Scotland possessed *tccP2*, the majority (13/15, 87%) of VTEC O26 isolated from patients in continental Europe carried this gene (Table 3.13). Among the 22 VTEC O26 isolates recovered from Scottish cattle only eight (36%) harboured *tccP2*; seven of these isolates possessed vtx_1 alone and only one carried vtx_1 in combination with vtx_2 (Table 3.13).

Investigation of the carriage of *tccP2* by *E. coli* O26 isolates possessing different sequences for the region upstream of *LEE1* found strong evidence of an association ($p < 0.001$) between the two. The vast majority (21/23, 91%) of isolates carrying allele 1 for the region upstream of *LEE1* harboured the *tccP2* gene and all of the isolates carrying alleles 3, 4, 5 or 6 contained this gene. In contrast, the majority (19/30, 63%) of isolates carrying allele 2 did not possess *tccP2*.

Table 3.13. Prevalence of *tccP2* among *E. coli* O26 isolates from different origins with different verocytotoxin genotypes.

Origin of isolate, verocytotoxin genotype	Number of isolates	Number of isolates positive for *tccP2*
Human (Scotland)		
vtx neg	5	5
vtx_1	3	0
vtx_1vtx_2	2	0
Human (continental Europe)		
vtx neg	4	4
vtx_1	5	4
vtx_2	6	5
vtx_1vtx_2	4	4
Cattle		
vtx neg	9	9
vtx_1	15	7
vtx_1vtx_2	7	1

3.4.1 Tir typing of *E. coli* O26 isolates

In addition to examining the carriage of *tccP* and *tccP2* among the *E. coli* O26 isolates, it was also determined whether the isolates possess an EHEC O157:H7 (EDL933)-like Tir (that is, a serine at reside 478) or an EPEC O127:H6 (E2348/69)-like Tir that can undergo tyrosine (Y474) phosphorylation and utilise the Nck actin polymerisation pathway. Discriminatory PCR, using gene-specific forward primers with a conserved reverse primer, revealed that all *E. coli* O26 isolates contained an EPEC-like Tir.

3.4.2 Summary of *tccP* / *tccP2* carriage results

None of the *E. coli* O26 isolates carried the *tccP* gene whereas the majority (62%) harboured *tccP2*. The three LEE-negative isolates did not possess the *tccP2* gene. *tccP2* exhibited size polymorphism among the isolates and it appeared that *E. coli* O26 isolates from patients in continental Europe carried smaller variants than those from patients in Scotland.

There was no evidence of an association between the carriage of *tccP2* and the ability to cause more serious clinical disease. All of the *vtx*-negative isolates, regardless of country of origin or host, carried *tccP2*. None of the VTEC O26 isolates from patients in Scotland harboured *tccP2* whereas the majority of such isolates recovered from patients in continental Europe carried this gene. Only 36% of Scottish cattle VTEC O26 isolates carried the *tccP2* gene.

3.5 Discussion

VTEC serogroup O26 is an important human pathogen in continental Europe, frequently being associated with life-threatening HUS (Caprioli *et al.*, 1997; Gerber *et al.*, 2002; Tozzi *et al.*, 2003; Zhang *et al.*, 2000). This is in contrast to the situation in Scotland and the rest of the United Kingdom where human infections associated with *E. coli* O26 are uncommon and are generally associated with simple diarrhoea. The finding that VTEC O26 strains are common and widely dispersed in Scottish cattle (Pearce *et al.*, 2006) is concerning if these have the potential to cause serious human disease. This study characterised *E. coli* O26 isolates from different backgrounds with two main objectives. Firstly, the study investigated *E. coli* O26 isolates from human infections of varying severity in an attempt to identify characteristics that allow these strains to cause more serious disease. This analysis included the majority of *E. coli* O26 isolates recovered from patients in Scotland, most of which were associated with simple diarrhoea, and a selection of isolates from patients in Germany and Italy, deliberately chosen because they possessed various combinations of virulence genes and were associated with different clinical forms of disease, including HUS. Secondly, the study compared *E. coli* O26 isolates from cattle in Scotland to those recovered from human infections to investigate the potential of Scottish cattle isolates to cause human disease. The cattle isolates examined were from a previous study (Pearce *et al.*, 2006) and were selected because they possessed different combinations of virulence genes. Among those deliberately chosen, the majority carried the gene(s) for verocytotoxin as these are considered to be more pathogenic. This chapter described the genotypic characterisation of *E. coli* O26 isolates by MLST and the presence of virulence genes to identify genetic traits which may allow these strains to cause more serious clinical disease.

The genetic relationship between the 63 *E. coli* O26 isolates from different backgrounds was analysed by MLST and further genetic analysis of the isolates was achieved by PCR detection of virulence genes. MLST revealed two genetically distant clones among the collection of *E. coli* O26 isolates. While the vast majority of isolates belonged to ST complex 29, three of the isolates grouped into a different ST complex, ST complex 10. Interestingly, these three isolates were shown to be

LEE-negative and were close evolutionary relatives to the non-pathogenic *E. coli* K-12 MG1655. It is possible that these isolates acquired the O26 O-antigen gene cluster by homologous recombination since previous research has shown that O-antigen gene clusters of different *E. coli* strains have conserved flanking regions (Wang & Reeves, 1998). Such a recombination event has been proposed to account for strains with very similar genotypes but different serotypes (Adiri *et al.*, 2003; Coffey *et al.*, 1998; Stine *et al.*, 2000). This demonstrates that the O serogroup cannot be considered as an indicator of genetic relatedness for *E. coli*. Furthermore, we should be cautious to draw conclusions about the pathogenic potential of *E. coli* O26 strains based on their serogroup alone.

The majority of the *E. coli* O26 isolates examined only differed by a single nucleotide in the *adk* locus and were resolved into two STs, ST21 and ST29. Despite the close genetic relationship between strains of ST21 and ST29, strains belonging to ST21 may have an increased likelihood of causing serious disease following human infection. This is interesting since previous research has found an association between MLST genotype and virulence potential. The majority of cases of invasive meningococcal disease are attributed to a small number of hypervirulent clones of *N. meningitidis* (Jolley *et al.*, 2000; Maiden *et al.*, 1998) and certain clones of *S. pneumoniae* appear to be more virulent than others, although the close correlation between MLST genotype and serotype means it is possible that serotype was a more important marker of virulence potential than genotype in this instance (Brueggemann *et al.*, 2003). These results are in contrast to a study of *S. aureus* which found no evidence of a correlation between MLST genotype and virulence potential (Feil *et al.*, 2003).

The finding that ST21 isolates may have a greater virulence potential is perhaps not surprising since the principal marker of strains belonging to ST21 was the possession of the *vtx* gene (either vtx_1, vtx_2 or vtx_1 and vtx_2 in combination), characterising them as typical VTEC. Only one isolate which was negative for *vtx* was grouped with these VTEC strains by MLST. It is possible that this isolate (which was recovered from a patient in Germany) lost its phage-borne *vtx* gene(s) during the course of

infection since previous studies observed a rapid loss of *vtx* in a subset of EHEC strains during the course of HUS (Bielaszewska *et al.*, 2007; Mellmann *et al.*, 2005; Mellmann *et al.*, 2008). However, it is also possible that this strain lost *vtx* during subculture as reported for other VTEC (Bielaszewska *et al.*, 2007; Karch *et al.*, 1992; Schmidt *et al.*, 1999b).

In this study, the *E. coli* O26 strains belonging to ST29 were generally *vtx*-negative and were thus characterised as atypical EPEC (aEPEC). The two exceptions to this were vtx_2-positive isolates recovered from patients in Germany. It is possible that these strains were aEPEC but recently acquired the vtx_2 phage since the ability of aEPEC to gain vtx_2-harbouring bacteriophages has been demonstrated previously (Bielaszewska *et al.*, 2007). The association between *vtx* gene carriage and the separation of *E. coli* O26 isolates into ST21 and ST29 observed in this study was corroborated by investigation of the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli). Analysis of the *E. coli* O26 strains that had their "pathogen type" entered when they were submitted to the database revealed that 75% (9 of 12) of the strains belonging to ST21 were classified as EHEC or VTEC and 92% (11 of 12) of the ST29 strains were EPEC.

MLST analysis in this study identified minimal variation in the nucleotide sequences of housekeeping genes among *E. coli* O26 isolates. Variations in nucleotide sequence of housekeeping genes have been observed previously among *E. coli* O78 strains (Adiri *et al.*, 2003) and among strains within a single serotype of other bacterial species (Feil *et al.*, 2000; Maiden *et al.*, 1998; Stine *et al.*, 2000). However, a previous study performed MLST on strains of *E. coli* O26:H11 and found very little genetic diversity (Gilmour *et al.*, 2005). This result is likely to be due to the fact that all of the strains analysed were isolated from human patients and harboured vtx_1 alone, with the exception of one strain which carried vtx_1 and vtx_2. Interestingly, MLST does not identify any sequence variation in housekeeping genes of *E. coli* O157:H7 isolates. MLST analysis on 77 *E. coli* O157:H7 isolates demonstrated that all strains had identical sequences for the seven housekeeping genes examined

(Noller *et al.*, 2003). Furthermore, all *E. coli* O157:H7 strains in the *E. coli* MLST database have an identical allelic profile and belong to ST11 (Wirth *et al.*, 2006).

Nucleotide sequencing of housekeeping genes found very little genetic variation in the genomic 'backbone' among the majority of *E. coli* O26 isolates, which corroborates the findings of Anjum *et al*. (2003). This indicated that closely related strains reside in patients from different countries and in different hosts. To look for greater genetic discrimination of the isolates, the nucleotide sequence of two additional targets, which may have a higher degree of diversity, was determined. Although the nucleotide sequences of *espA* in all but one of the *E. coli* O26 isolates were identical, differences in the sequence of the region upstream of *LEE1* were identified. The six different sequences discovered were assigned allele numbers 1 to 6. Alleles 1 and 2 were the most prevalent among the isolates and were carried by 38% and 50% of the isolates, respectively. The observations that allele 2 was only discovered in isolates belonging to MLST ST21 (or the closely related ST574); alleles 3 to 6 were only detected in isolates belonging to ST29; and allele 1 was present in both ST21 and ST29 isolates correlated with the results of *vtx* gene carriage by isolates possessing different alleles for the region upstream of *LEE1*. That is, while allele 2 was only detected in *vtx*-positive isolates and alleles 3 to 6 were only discovered in *vtx*-negative isolates, isolates carrying allele 1 were either *vtx*-positive or *vtx*-negative.

Importantly, the nucleotide sequences for the region upstream of *LEE1* begin to differentiate *E. coli* O26 isolates recovered from different backgrounds. While the majority of human isolates recovered in continental Europe carried allele 1, most of the isolates from patients and cattle in Scotland harboured allele 2. Notably, all of the *vtx*-positive Scottish human isolates and the vast majority of the *vtx*-positive Scottish cattle isolates carried allele 2. Only *vtx*-negative isolates recovered from patients and cattle in Scotland and two *vtx*-positive cattle isolates were found to possess allele 1. In contrast, only a small number of the *vtx*-positive isolates from patients in continental Europe carried allele 2. The majority of all human isolates from continental Europe harboured allele 1.

Since MLST found little genetic variation in the genomic 'backbone' among the majority of *E. coli* O26, the differences in pathogenic potential between isolates is presumed to be a consequence of additional virulence genes that have inserted into their genome. Indeed, PFGE analysis has proven that there is considerable heterogeneity in the O26 genome (L. Allison, personal communication; Zhang *et al.*, 2000). Therefore, the presence of the genes encoding the virulence factors Vtx1, Vtx2, LEE and enterohaemolysin was determined for each *E. coli* O26 isolate by PCR, and associations between the virulence factors possessed and the severity of disease elicited by the human isolates were considered. The production of Vtx is a major virulence determinant of *E. coli* strains. The majority of the isolates associated with diarrhoea were *vtx*-negative. Of the five isolates which were *vtx*-positive, four possessed the vtx_1 gene alone and only one isolate carried both vtx_1 and vtx_2. No isolate associated with diarrhoea harboured the vtx_2 gene alone. All of the isolates associated with HUS were *vtx*-positive; 3/12 possessed vtx_1 alone, 5/12 possessed vtx_2 alone and 4/12 possessed vtx_1 and vtx_2. While a reasonable number of the isolates associated with HUS carried vtx_1 as their sole *vtx* gene this is not representative of the situation at large. Two of the three isolates originated from Italy although very few Vtx1-producing strains have ever been isolated from cases of human infection, particularly from severe cases, in Italy (S. Morabito, personal communication). These isolates were originally included in this study to identify additional factor(s) possibly responsible for their increased pathogenicity. It was not possible to perform statistical analysis on these results since the European isolates were deliberately chosen because they possessed different combinations of *vtx* genes associated with different forms of disease. However, previous research has shown that the presence of the vtx_2 gene in the infecting strain is strongly associated with severe disease in humans (Boerlin *et al.*, 1999; Brooks *et al.*, 2005; Friedrich *et al.*, 2002). Furthermore, the administration of purified Vtx2, but not of Vtx1, has been shown to cause HUS in experimentally treated primates (Siegler *et al.*, 2003).

A further virulence factor of certain *E. coli* strains is the presence of the LEE pathogenicity island which carries the genes required for the formation of A/E lesions. Carriage of the LEE was determined by detecting two genes encoded on

separate operons within the pathogenicity island (*eae* in the *LEE5* operon and *sepL* in the *LEE4* operon). There was an absolute correlation between the presence of *eae* and the presence of *sepL*, suggesting that either gene could be used alone when determining the carriage of the LEE. A previous study corroborates this finding by showing an absolute correlation between the presence of *eae* and the presence of eight other genes of the LEE (Bertin *et al.*, 2004). The LEE was present in all but one of the human isolates analysed, recovered from patients suffering from disease of varying severity, ranging from simple diarrhoea to life-threatening HUS. A close association between infections with LEE-positive VTEC and severe disease in humans has been demonstrated previously (Boerlin *et al.*, 1999; Jenkins *et al.*, 2003). However, it has also been shown that certain LEE-negative strains of VTEC were associated with bloody diarrhoea and HUS (Boerlin *et al.*, 1999; Bonnet *et al.*, 1998), indicating that the LEE is not essential for the virulence of certain VTEC for humans.

The *hlyA* gene was detected in 9/14 isolates associated with diarrhoea, 2/3 isolates associated with bloody diarrhoea or haemorrhagic colitis and 8/12 isolates associated with HUS. This suggests that enterohaemolysin is not a major virulence factor involved in severe human disease. Similarly, a previous study found no association between the presence of the *hly* gene and HUS (Jenkins *et al.*, 2003). However, enterohaemolysin from *vtx*-negative *E. coli* O26 isolates associated with HUS was shown to be responsible for the microvascular endothelial cell cytotoxicity of these strains (Aldick *et al*., 2007). This suggested that enterohaemolysin may also cause endothelial damage during infection and contribute to the progression to HUS (Aldick *et al*., 2007). In addition, patients suffering from HUS have been shown to have a specific immune response against enterohaemolysin (Schmidt *et al.*, 1995).

Since one of the isolates, recovered from a patient in Scotland suffering from diarrhoea, did not possess any of the virulence factors screened for, it suggests that occasionally the disease process may involve the possession of unknown virulence factors or perhaps exquisite host susceptibility.

The ability of *E. coli* strains to form A/E lesions contributes to their pathogenicity by promoting colonisation of the intestinal mucosa. A characteristic feature of the A/E lesion is the formation of an actin-rich pedestal-like structure in the host cell underneath the membrane at the site of attachment. Until recently, it was widely believed that EHEC and EPEC induce actin polymerisation by distinct mechanisms. Tir EPEC O127:H6 requires tyrosine (Y474) phosphorylation and the N-WASP activator protein Nck, whereas Tir EHEC O157:H7 utilises the N-WASP activator TccP. However, analysis of a large number of non-O157 EHEC and EPEC strains has broken the prevailing dogma and it appears that many EPEC and EHEC strains have the ability to use both the Nck and TccP pathways to trigger actin polymerisation (Garmendia *et al.*, 2005b; Ogura *et al.*, 2007; Ooka *et al.*, 2007; Whale *et al.*, 2006; Whale *et al.*, 2007). TccP2 is functionally equivalent to TccP and the gene which encodes TccP2 (*tccP2*) has been detected in SF EHEC O157:NM and the majority of non-O157 EHEC (serogroups O26, O103, O111, O121 and O145) (Ogura *et al.*, 2007).

This study found all LEE-positive *E. coli* O26 isolates contained an EPEC-like Tir so presumably triggering actin polymerisation using the Nck pathway, although functional studies would need to be done to confirm this. The carriage of *tccP* and *tccP2* among the LEE-positive isolates was determined by PCR. None of the sixty isolates contained the *tccP* gene. Although sequence variation could explain why the primers may not amplify all *tccP* variants, it should be noted that the primers used have successfully amplified *tccP* from *E. coli* O26 in a previous study (Garmendia *et al.*, 2005b). Indeed, Garmendia *et al*. (2005b) found *tccP* in 5 of 27 (18.5%) EHEC O26 strains analysed, although none of the eleven EPEC O26 isolates examined carried the gene.

The majority (65%) of the LEE-positive *E. coli* O26 isolates investigated in this study contained *tccP2*. As has been noted in previous studies, *tccP2* exhibited size polymorphism among the isolates analysed, ranging from 750 bp to 1,400 bp. Different sized *tccP2* genes are likely to encode polypeptides which vary in the number of PRRs. Generally, it appeared that *E. coli* O26 isolates from clinical cases

in continental Europe contained smaller *tccP2* variants than those isolated from patients in Scotland. A previous study showed similar results in that the VTEC O26 of human origin isolated in the UK carried a larger *tccP2* variant (1,200 bp) than isolates recovered in continental Europe (1,050 bp and smaller) (Ogura *et al.*, 2007). A recent functional study has shown that, although TccP consisting of the N-terminal translocation domain and two PRRs is both necessary and sufficient for actin polymerisation, there is a direct correlation between the number of PRRs and the affinity of TccP to N-WASP (Garmendia *et al.*, 2006). However, it is presently not known if there is an association between the number of PRRs and the efficiency of actin pedestal formation during infection.

It is interesting to note that one of the isolates (ZAP1125_G17) appeared to carry two copies of *tccP2*, which differed in the number of PRRs they encode. Although such an observation has not been reported previously, it is possible that other strains investigated harbour multiple copies of the gene which are the same size and will not be distinguished by PCR. Southern blotting could be employed to determine the number of copies of *tccP2* carried by the isolates.

The majority (79%) of the clinical isolates carried *tccP2* but there was no evidence of an association between the carriage of *tccP2* and the ability to cause more serious clinical disease. This is in part due to the fact that a large number of the diarrhoea cases were associated with aEPEC isolates and this study found that 100% of the aEPEC isolates carried the *tccP2* gene. A previous study looking at a larger number of isolates also revealed that the vast majority (82%) of aEPEC O26 harboured *tccP2* (Ooka *et al.*, 2007). aEPEC are likely to rely on A/E lesion formation as a major virulence trait. Therefore, it may be advantageous for the bacterium to express TccP2 if co-translocation of Tir with its adaptor protein increases the efficiency of A/E lesion formation and colonisation, as has been suggested previously (Garmendia *et al.*, 2005b). It is interesting that the carriage of *tccP2* is different between VTEC O26 clinical isolates from Scotland and continental Europe. None of the VTEC O26 isolates recovered from humans in Scotland harboured *tccP2*, whereas the majority (87%) of VTEC O26 isolated from clinical cases in continental Europe carried the

gene. It is possible that the *tccP2*-negative Scottish clinical VTEC O26 isolates belong to a particular "Scottish" clone because previous examination of a single VTEC O26 of human origin isolated in the UK revealed that it was *tccP2* positive (Ogura *et al.*, 2007). The possession of *tccP2* by the majority of VTEC O26 isolates recovered in Germany and Italy corroborates previous findings (Ogura *et al.*, 2007).

It is interesting to note that there is an association between the carriage of *tccP2* by *E. coli* O26 isolates and the nucleotide sequence for the region upstream of *LEE1*. The fact that almost all of the isolates carrying allele 1 for the region upstream of *LEE1* harboured *tccP2* can be explained by the fact allele 1 is carried by many of the *vtx*-negative isolates and the majority of European human isolates, both of which have been found to be associated with the carriage of *tccP2*. Furthermore, the fact that all of the isolates carrying alleles 3, 4, 5 or 6 harboured *tccP2* is likely to be due to the fact that these alleles are only carried by *vtx*-negative isolates. Finally, the fact that the majority of isolates carrying allele 2 did not possess *tccP2* can be explained by the fact allele 2 is carried by all VTEC O26 from patients in Scotland and the majority of VTEC O26 from Scottish cattle, both of which generally lack the *tccP2* gene.

Molecular typing data suggests that the majority of VTEC O26 from clinical cases in continental Europe and all aEPEC O26, regardless of background, have the potential to trigger actin polymerisation by both the Nck and TccP2 pathways. However, functional studies would need to be done to confirm this. The ability of *E. coli* O26 isolates to be bimodal for actin polymerisation confirms the importance of A/E lesion formation to these pathogens and perhaps the possession of TccP2 may offer these strains a competitive advantage in the environment, facilitating their survival and transmission.

The fact that the majority of the *E. coli* O26 isolates recovered from patients in Scotland were *vtx*-negative or carried vtx_1 alone begins to explain why, in Scotland, human infections associated with *E. coli* O26 generally only result in simple diarrhoea. In continental Europe, the occurrence of *E. coli* O26 strains which

harbour *vtx*$_2$, either alone or in combination with *vtx*$_1$, serves as one explanation why it is experiencing serious human infection. Although MLST analysis identified that the *E. coli* O26 isolates associated with human infection in Scotland and continental Europe were very closely related, the importance of *vtx* genes in strains associated with severe human disease indicates that the ability of strains to cause serious human infection depends more on horizontally acquired DNA than on the vertically inherited genotype.

Furthermore, despite the initial observation that *E. coli* O26 isolates recovered from patients in Scotland and continental Europe were closely related, differences between these two groups of isolates emerged following investigation of the nucleotide sequence for the region upstream of *LEE1* and the carriage of *tccP2*. Although the aEPEC isolates from both groups were very similar, there were clear differences between the VTEC isolates. While all the VTEC O26 isolates recovered from patients in Scotland carried allele 2 for the region upstream of *LEE1* and were negative for *tccP2*, the majority of the VTEC O26 isolates from patients in continental Europe carried allele 1 and were positive for *tccP2*. Although this would suggest that the carriage of allele 1 and *tccP2* are indicators of greater virulence potential, this may not be the case since these are also traits of the aEPEC isolates which are generally associated with mild clinical disease. However, if this genotype is in a VTEC background, particularly in a strain harbouring *vtx*$_2$, there may be a strong possibility that this strain could cause severe human disease.

MLST analysis revealed that the *E. coli* O26 strains residing in cattle in Scotland were closely related to the pathogenic human isolates. This suggests that there is a risk of cattle isolates becoming important human pathogens if they possess the necessary virulence determinants. The majority (51%) of *E. coli* O26 shed by cattle in Scotland are *vtx*-negative and among those which are *vtx*-positive, most (75%) harboured *vtx*$_1$ alone (Pearce *et al.*, 2006). No Scottish cattle isolate possessed *vtx*$_2$ alone (Pearce *et al.*, 2006). In addition, my study found that the majority of VTEC O26 isolates recovered from Scottish cattle carried allele 2 for the region upstream of *LEE1* and were negative for *tccP2*. Therefore, it appears that the majority of Scottish

cattle isolates have different genotypic characteristics to those causing serious human disease and this could explain the low incidence of *E. coli* O26 infections in Scotland. However, it could be concerning that one of the Scottish cattle isolates carried vtx_1, vtx_2, LEE, *hlyA*, *tccP2* and allele 1 for the region upstream of *LEE1*, and was thus indistinguishable from an isolate recovered from a case of HUS in continental Europe.

Chapter 4

Phenotypic characterisation of *Escherichia coli* O26 isolates

Although knowledge of the complement of virulence genes harboured by a strain is important, an understanding of the ability of the organism to express them is also essential. Phenotypic characterisation assays were performed on *E. coli* O26 clinical isolates to give a read out of expression differences between isolates, with the aim of identifying traits which allow strains to cause more serious clinical disease. In addition, phenotypic analysis of *E. coli* O26 isolates recovered from Scottish cattle may indicate the potential of bovine isolates to cause human infection. This chapter describes the phenotypic characterisation of human and bovine *E. coli* O26 isolates by Vero cell cytotoxicity assays to investigate levels of verocytotoxin production; LEE secreted protein analysis; and adherence assays to Caco-2 cells.

4.1 Vero cell cytotoxicity assays

The production of verocytotoxin (Vtx) is a major virulence trait of VTEC and is thought to be the principal factor responsible for the serious complications of VTEC infection (World Health Organisation, 1998). A number of observations indicate that the amount of Vtx produced by infecting VTEC strains may correlate with the clinical outcome of infection (Eklund *et al.*, 2002; Muniesa *et al.*, 2003). Therefore, it was investigated whether increased Vtx production by certain VTEC O26 isolates could account for why they were associated with more serious disease. This work also examined whether Scottish cattle isolates produced comparable amounts of Vtx to human isolates. Analysis of Vtx production was achieved by determining the Vtx titres in culture filtrates from a subset of the VTEC O26 isolates using the Vero cell cytotoxicity assay. A total of thirty VTEC O26 isolates were examined. Twelve isolates carried vtx_1 alone, six harboured vtx_2 alone and twelve possessed vtx_1 and vtx_2. Among the strains containing either vtx_1 or vtx_1 and vtx_2, an equal number of isolates from cattle and cases of human infection were analysed.

The basal level of Vtx production by each isolate was assessed by culturing the isolates in DMEM (iron-replete) for 24 h. The Vtx titre in the culture filtrate of each isolate was determined using the results from two or three independent experiments (Table 4.1).

Table 4.1. Verocytotoxin (Vtx) titres of VTEC O26 isolates cultured in DMEM by Vero cell cytotoxicity assay.

	Isolate[a]	Vtx titre[b]		
		Range	Geometric mean	Median
vtx$_1$ gene	ZAP1079_B3	64	64	64
	ZAP1081_B5	16 to 64	32	40
	ZAP1088_B12	8 to 64	23	36
	ZAP1095_B19	8 to 64	23	36
	ZAP1103_B27	4 to 32	11	18
	ZAP1107_B31	16 to 64	32	32
	ZAP1114_S6 [D]	8 to 64	20	16
	ZAP1115_S7 [D]	32 to 64	45	48
	ZAP1124_G16 [HUS]	32 to 64	45	48
	ZAP1127_G19 [D]	64	64	64
	ZAP1146_I29 [HUS]	256 to 512	362	384
	ZAP1151_I34 [HUS]	64	64	64
vtx$_2$ gene	ZAP1128_G20 [HUS]	<2 to 2	<2	2
	ZAP1132_G24 [HUS]	<2 to 8	2	<2
	ZAP1134_G26 [HUS]	<2	<2	<2
	ZAP1147_I30 [HC]	<2	<2	<2
	ZAP1149_I32 [HUS]	<2	<2	<2
	ZAP1150_I33 [HUS]	<2	<2	<2
vtx$_1$ and *vtx*$_2$ genes	ZAP1078_B2	16 to 32	20	16
	ZAP1086_B10	<2 to 16	4	8.5
	ZAP1094_B18	32	32	32
	ZAP1100_B24	<2 to 32	6	16.5
	ZAP1106_B30	16 to 64	32	32
	ZAP1109_B33	2 to 16	6	9
	ZAP1122_G14 [HUS]	64 to 256	128	128
	ZAP1126_G18 [HUS]	64	64	64
	ZAP1129_G21 [HUS]	16 to 64	32	40
	ZAP1130_G22 [HUS]	<2 to 64	8	32.5
	ZAP1152_S35 [D]	4 to 128	23	66
	ZAP1153_S36 [BD]	<2 to 32	6	16.5

[a] The associated clinical disease is indicated in parenthesis: D, diarrhoea; BD, bloody diarrhoea; HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome.

[b] The Vtx titre was defined as the reciprocal of the highest dilution of the culture filtrate that caused a cytotoxic effect in 50% of the Vero cells after 3 days of incubation. The Vtx titre in the culture filtrate of each isolate was determined using the results from at least two independent experiments.

While isolates possessing a particular *vtx* genotype generally had similar Vtx titres, the culture filtrate from the vtx_1-positive isolate ZAP1146_I29 had a higher Vtx titre than the culture filtrates from the other isolates harbouring vtx_1 (Table 4.1). With the exception of isolate ZAP1146_I29 which was recovered from a patient with HUS and had a higher Vtx titre, the Vtx titres from Vtx1-producing isolates recovered from cases of diarrhoea and HUS were comparable (Table 4.1). In addition, the Vtx titres of isolates positive for vtx_1 and vtx_2 recovered from diarrhoea, bloody diarrhoea and HUS infections were also similar (Table 4.1).

Comparison of the Vtx titres of VTEC O26 isolates that harboured different *vtx* genotypes showed that culture filtrates from isolates carrying vtx_1 alone and vtx_1 in combination with vtx_2 were cytotoxic to Vero cells (Table 4.2). The culture filtrates from isolates that possessed both vtx_1 and vtx_2 had slightly reduced cytotoxicty to Vero cells than those from isolates that carried vtx_1 alone (Table 4.2), but this difference was not statistically significant. The culture filtrates from isolates harbouring vtx_2 alone had no cytotoxic activity on Vero cells (Table 4.2). Investigation of Vtx production by VTEC O26 isolates recovered from cases of human infection found that, among the isolates harbouring either vtx_1 alone or both vtx_1 and vtx_2, the culture filtrates from isolates recovered in continental Europe tended to have a greater Vtx titre than those from isolates recovered in Scotland with the same *vtx* genotype (Table 4.2). However, these differences were not found to be statistically significant. The culture filtrates from Scottish cattle VTEC O26 isolates carrying vtx_1 alone and both vtx_1 and vtx_2 had a comparable Vtx titre to those from VTEC O26 isolates from clinical cases in Scotland, but a lower Vtx titre than those from isolates recovered from patients in continental Europe, with the same *vtx* genotype (Table 4.2).

Table 4.2. Verocytotoxin (Vtx) titres of VTEC O26 isolates with different *vtx* genotypes from different origins using the Vero cell cytotoxicity assay.

***vtx* genotype, origin of isolate**	**No. of isolates**	**Vtx titre[a]**		
		Range	**Geometric mean**	**Median**
vtx_1	12	4 to 512	38.9	64
Human (Scotland)	2	8 to 64	27.9	32
Human (cont. Europe)	4	32 to 512	95.1	64
Cattle	6	4 to 64	27.3	32
vtx_2	6	<2 to 8	<2	<2
vtx_1 and vtx_2	12	<2 to 256	19.1	32
Human (Scotland)	2	<2 to 128	11.3	18
Human (cont. Europe)	4	<2 to 256	43.5	64
Cattle	6	<2 to 64	13.1	16

[a] The Vtx titre was defined as the reciprocal of the highest dilution of the culture filtrate that caused a cytotoxic effect in 50% of the cells in the Vero cell monolayer after 3 days of incubation.

The level of Vtx production by each isolate was also determined following the culture of the isolates in the presence of mitomycin C and in low-iron medium. Mitomycin C has been shown to induce *vtx* expression (al-Jumaili *et al.*, 1992; Ritchie *et al.*, 2003) and low-iron concentrations can increase Vtx1 production (Ritchie *et al.*, 2003). Initial experiments made two-fold serial dilutions of culture filtrates in a single 96-well microtitre plate and the maximum dilution prepared was 1:4,096. In many cases the inducing conditions increased Vtx titres to values in excess of 4,096 and thus no definitive values were obtained. Additional experiments preparing dilutions of culture filtrates up to 1:16,777,216 were performed on certain isolates. For many of the isolates there was at least one Vtx titre under both of the inducing conditions that was not a definitive value and, therefore, it was not possible to calculate an average Vtx titre for each isolate. However, since every condition (non-induced, in the presence of mitomycin C and in low-iron medium) for any one isolate was examined in the same experiment, it was possible to select a representative data set for each isolate (Table 4.3). Definitive Vtx titres were obtained for all the isolates examined harbouring vtx_1 alone and vtx_2 alone which

allowed the effect of culturing these isolates under different inducing conditions to be analysed.

Culturing the VTEC O26 isolates in the presence of mitomycin C increased the Vtx titre in the culture filtrates of all isolates with the exception of ZAP1128_G20, which showed no change in its Vtx titre (Table 4.3). There was a significant increase ($P < 0.001$) in the Vtx titres of the cattle isolates harbouring *vtx*$_1$ alone following mitomycin C treatment. Increases of 7-fold or greater were observed for five of six of these isolates (median fold increase = 8.5) (Figure 4.1). In contrast, mitomycin C treatment of human isolates that carried *vtx*$_1$ alone resulted generally in a lower increase in the Vtx titres (median fold increase = 4) (Figure 4.1). The exception to this was isolate ZAP1127_G19, which had a dramatic increase (18-fold) in its Vtx titre following mitomycin C treatment. Although Vtx titres increased more for the cattle isolates than for the human isolates harbouring *vtx*$_1$ in response to mitomycin C treatment, there was no significant difference in the Vtx titres between these two groups following mitomycin C induction (median Vtx titres for the cattle and human isolates were 2048 and 1024, respectively). There was no evidence of an association between the level of increase in Vtx titres by the *vtx*$_1$-positive human isolates following mitomycin C treatment and the severity of disease.

Culturing the VTEC O26 isolates which carried *vtx*$_2$ alone in the presence of mitomycin C increased the Vtx titres in the culture filtrates from the majority (5 of 6) of the isolates to levels which were cytotoxic to Vero cells (Table 4.3). However, these culture filtrates exhibited a much lower cytotoxic activity on Vero cells compared to those from mitomycin C-induced VTEC O26 isolates that harboured solely *vtx*$_1$ or both *vtx*$_1$ and *vtx*$_2$ (Table 4.3; median Vtx titres for VTEC O26 strains carrying *vtx*$_2$ alone and *vtx*$_1$ alone following mitomycin C treatment were 24 and 1536, respectively).

Table 4.3. Verocytotoxin (Vtx) titres of VTEC O26 isolates cultured under different conditions by Vero cell cytotoxicity assay.

		Vtx titre[b]		
	Isolate[a]	Non-induced[c]	Mitomycin C[d]	Low-iron[e]
vtx$_1$ gene	ZAP1079_B3	64	1024	1024
	ZAP1081_B5	16	8192	16,777,216
	ZAP1088_B12	8	1024	524,288
	ZAP1095_B19	8	2048	131,072
	ZAP1103_B27	4	2048	8192
	ZAP1107_B31	16	8192	2,097,152
	ZAP1114_S6 [D]	16	128	32,768
	ZAP1115_S7 [D]	32	1024	1,048,576
	ZAP1124_G16 [HUS]	32	512	16,384
	ZAP1127_G19 [D]	64	16,777,216	16,777,216
	ZAP1146_I29 [HUS]	512	4096	65,536
	ZAP1151_I34 [HUS]	64	1024	4096
vtx$_2$ gene	ZAP1128_G20 [HUS]	2	2	16
	ZAP1132_G24 [HUS]	<2	64	8
	ZAP1134_G26 [HUS]	<2	8	<2
	ZAP1147_I30 [HC]	<2	32	<2
	ZAP1149_I32 [HUS]	<2	16	8
	ZAP1150_I33 [HUS]	<2	64	16
vtx$_1$ and *vtx*$_2$ genes	ZAP1078_B2	32	1024	>4096
	ZAP1086_B10	16	>4096	>4096
	ZAP1094_B18	32	>4096	>4096
	ZAP1100_B24	32	>4096	>4096
	ZAP1106_B30	16	16,384	131,072
	ZAP1109_B33	16	>4096	1024
	ZAP1122_G14 [HUS]	256	>4096	>4096
	ZAP1126_G18 [HUS]	64	>4096	>4096
	ZAP1129_G21 [HUS]	16	512	>4096
	ZAP1130_G22 [HUS]	64	512	>4096
	ZAP1152_S35 [D]	128	>4096	>4096
	ZAP1153_S36 [BD]	32	>4096	>4096

[a] The associated clinical disease is indicated in parenthesis: D, diarrhoea; BD, bloody diarrhoea; HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome.

[b] The Vtx titre was defined as the reciprocal of the highest dilution of the culture filtrate that caused a cytotoxic effect in 50% of the Vero cells after 3 days of incubation. The Vtx titre values for each isolate were obtained from one representative experiment.

[c] Isolates were cultured in DMEM (iron-replete).

[d] Isolates were cultured in DMEM (iron-replete) in the presence of mitomycin C (0.5 µg/ml).

[e] Isolates were cultured in DMEM with a low-iron concentration.

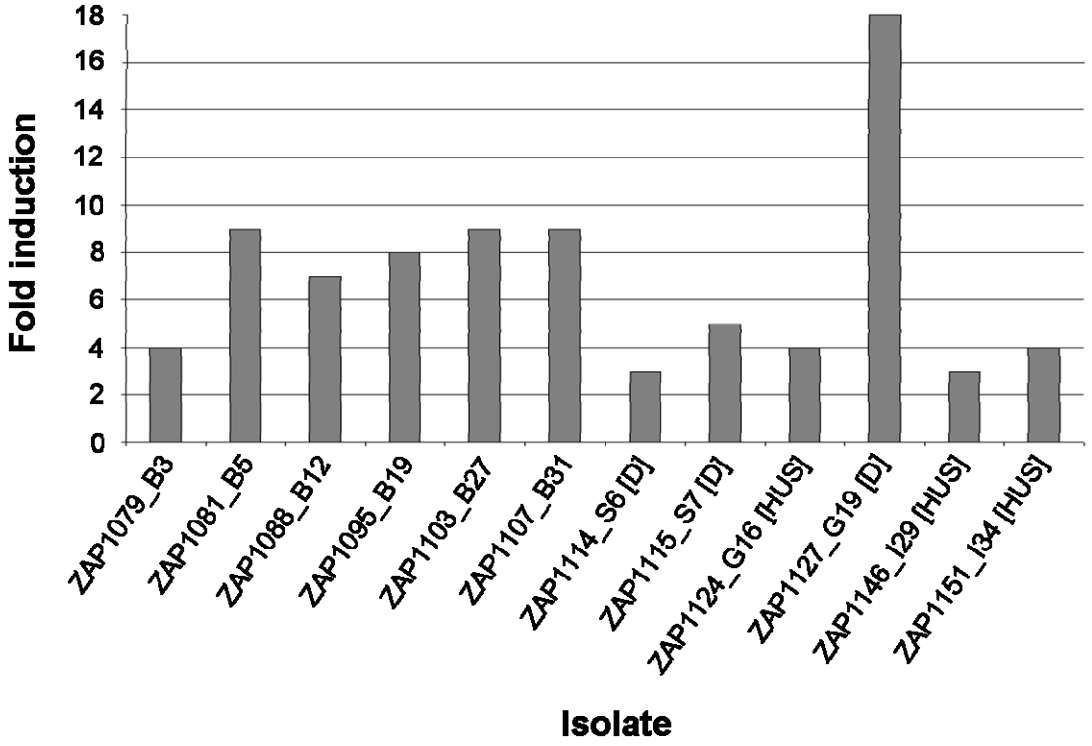


Figure 4.1. Increase in verocytotoxin (Vtx) titre following mitomycin C treatment of VTEC O26 isolates that harbour *vtx*$_1$ alone. The bars represent the fold change in Vtx titre of mitomycin C-treated cultures compared to non-induced cultures. The Vtx titre values for each isolate were obtained from one representative experiment and are detailed in Table 4.3. The clinical disease associated with each human isolate is indicated in parenthesis after the isolate number: D, diarrhoea; HUS, haemolytic uraemic syndrome.

Since low-iron concentrations have been shown previously to induce Vtx1 production (Ritchie *et al.*, 2003), it was not surprising that the Vtx titres were greater following culture in low-iron medium for all VTEC O26 isolates that carried the vtx_1 gene (Table 4.3). Moreover, the increase in Vtx titre from isolates harbouring vtx_1 alone was significantly greater (P = 0.01) following growth in low-iron medium (median fold increase = 12.5) than in the presence of mitomycin C (median fold increase = 6). Culturing the vtx_1-positive isolates in low-iron medium resulted in variable increases in Vtx titres among both the cattle and human isolates (Figure 4.2).

Similar to the situation following mitomycin C treatment, the median fold increase in Vtx titre by the vtx_1-positive cattle isolates (median = 15) was greater than that by the vtx_1-positive human isolates (median = 10) in response to low-iron concentrations, although this difference was not statistically significant. There was a large difference in the Vtx titres between these two groups of isolates after culture in low-iron medium (median Vtx titres for the cattle and human isolates were 327,680 and 49,152, respectively) but, again, this difference was not statistically significant. The vtx_1-positive isolates recovered from patients with diarrhoea appeared to have greater increases in Vtx titres in response to low-iron concentrations than those recovered from patients with HUS (Figure 4.2).

Culture of the vtx_2-positive VTEC O26 isolates in low-iron medium increased the Vtx titres in the culture filtrates from four of the six isolates (Table 4.3). However, the median fold increase in Vtx titre from isolates harbouring vtx_2 alone was less in low-iron concentrations (median = 3) than following mitomycin C treatment (median = 4.5).

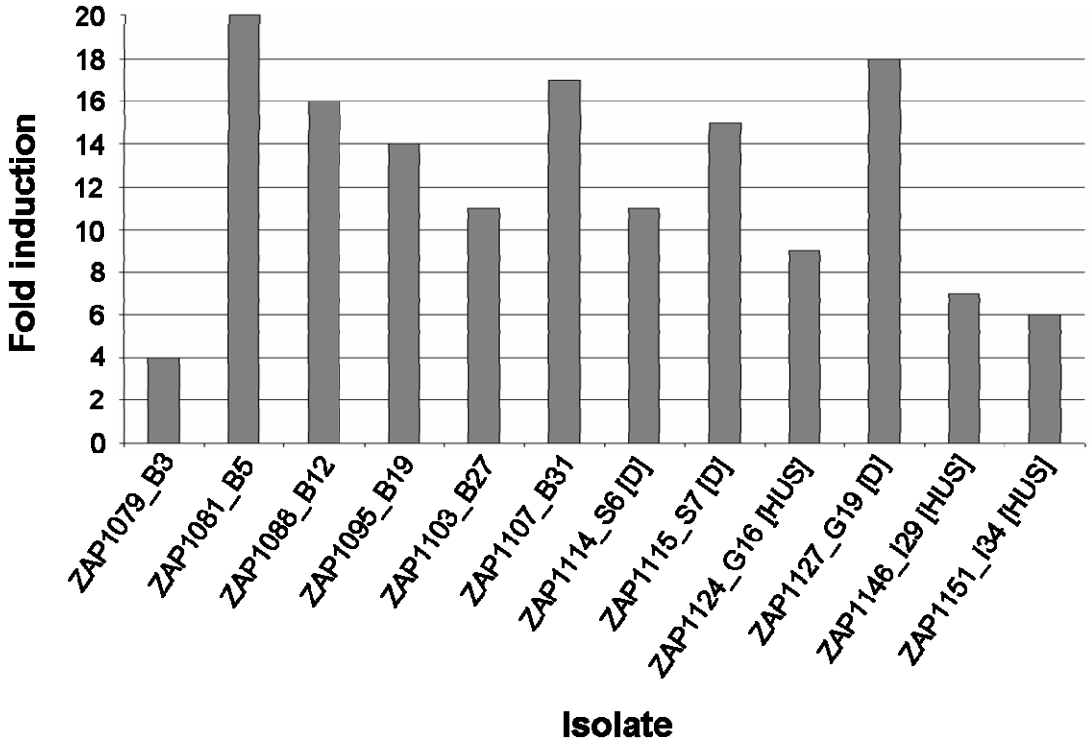


Figure 4.2. Increase in verocytotoxin (Vtx) titre following culture in low-iron medium of VTEC O26 isolates that harbour *vtx*$_1$ alone. The bars represent the fold change in Vtx titre of cultures grown in low-iron medium compared to non-induced cultures. The Vtx titre values for each isolate were obtained from one representative experiment and are detailed in Table 4.3. The clinical disease associated with each human isolate is indicated in parenthesis after the isolate number: D, diarrhoea; HUS, haemolytic uraemic syndrome.

An observation following the Vero cell cytotoxicity assays was that the culture filtrates from isolates harbouring *vtx*$_2$ alone had no or little cytotoxic activity on Vero cells. Since previous research has shown that VTEC strains, including those belonging to serogroup O26, can lose their verocytotoxin genes during subculture (Bielaszewska *et al.*, 2007; Karch *et al.*, 1992), the stability of the *vtx* genes in a selection of the isolates under the experimental culture conditions analysed was tested. To confirm the *vtx* genotype of the inoculum, a single colony was suspended in PBS and ten-fold dilutions inoculated onto LB agar. After overnight incubation, ten colonies from plates with approximately 100 well-separated colonies were PCR screened for *vtx*$_1$ and *vtx*$_2$. Following culture of the isolates under non-inducing conditions, in the presence of mitomycin C and in low-iron medium, ten-fold dilutions of the liquid cultures were inoculated onto LB agar and the presence of *vtx*$_1$ and *vtx*$_2$ genes in up to ten colonies were determined by PCR. None of the VTEC O26 isolates examined had lost their *vtx* gene(s) during culture. Therefore, the low or non-existent Vtx titres from isolates carrying *vtx*$_2$ alone cannot be explained by the loss of *vtx*$_2$ *in vitro*.

4.1.1 Summary of Vero cell cytotoxicity results

Vero cell cytotoxicity assays revealed that culture filtrates from VTEC O26 isolates harbouring *vtx*$_2$ alone had no or low cytotoxic activity on Vero cells whereas those from VTEC O26 isolates containing *vtx*$_1$, either alone or in combination with *vtx*$_2$, had significantly higher cytotoxic activity. The Vero cell assay also demonstrated that the culture filtrate from the non-induced *vtx*$_1$-positive isolate ZAP1146_I29 had a higher Vtx titre than the culture filtrates from other isolates harbouring *vtx*$_1$ alone and this isolate was recovered from a patient suffering from HUS. However, generally, there was no evidence of an association between greater Vtx titres and more serious clinical disease.

The culture filtrates from non-induced Scottish cattle VTEC O26 isolates carrying *vtx*$_1$ alone and both *vtx*$_1$ and *vtx*$_2$ had comparable Vtx titres to those from non-induced VTEC O26 isolates recovered from patients in Scotland, but lower Vtx titres to those from non-induced VTEC O26 isolates recovered from patients in continental

Europe, with the same *vtx* genotype. Mitomycin C treatment of the vtx_1-positive isolates increased Vtx titres more for the cattle isolates than for the human isolates and there was no significant difference in the Vtx titres between these two groups following mitomycin C induction.

4.2 LEE-encoded protein secretion

The ability of certain *E. coli* strains to form A/E lesions on intestinal epithelial cells is considered an important marker for their pathogenicity. The formation of A/E lesions is dependent on the LEE pathogenicity island. Following initial attachment of the bacterium to the host cell, type III secretion of Tir through an EspA filament and an EspB/EspD pore in the host cell membrane promotes intimate attachment via intimin and Tir (Frankel *et al.*, 1998). The continued production and secretion of LEE-encoded proteins results in host cell actin rearrangement and the formation of A/E lesions (Frankel *et al.*, 1998).

Previous research by the ZAP group has shown considerable variation in the levels of LEE-secreted proteins among *E. coli* O157 strains (Roe *et al*., 2003; unpublished results). The aim of this analysis was to examine if there are differences in the level of secretion of LEE-encoded proteins by *E. coli* O26 isolates. In particular, it was to investigate whether variations in the amount of LEE protein secretion could be detected between, (1) isolates associated with infections of different severity and (2) isolates from cases of human disease and from cattle. This characterisation was performed on the *E. coli* O26 isolates which were LEE-positive and carried the genes for both Vtx1 and Vtx2. Such strains were selected for analysis because they are the isolates present in Scottish cattle which, based solely on their *vtx* genotype, are considered most likely to cause serious human disease.

4.2.1 Level of secretion of LEE-encoded proteins

In order to determine the level of secretion of LEE-encoded proteins by *E. coli* O26 isolates, the proteins present in the supernatants of the thirteen isolates, seven bovine and six human, positive for both vtx_1 and vtx_2 genes were analysed. It has been shown that DMEM medium is permissive for type III secretion of EspA and EspB by EPEC and VTEC (Ebel *et al.*, 1996; Kenny & Finlay, 1995). Therefore, isolates were cultured in DMEM-HEPES to an OD_{600} of 0.8 and supernatant proteins were TCA precipitated, separated by SDS-PAGE and visualised by Colloidal blue staining. The isolates were cultured on three separate occasions and the secreted protein profiles in each instance are shown in Figure 4.3A.

Three prominent protein bands were observed in the majority of the samples. The protein of 66 kDa detected in all samples is BSA, which was added to the supernatants before precipitation to serve as a co-precipitant to maximise protein recovery. Similar levels of BSA were present in all protein samples (Figure 4.3A) which indicates that the precipitation procedure was efficient and allows comparison of protein levels between isolates. The secreted protein profiles of the VTEC O26 isolates are comparable to those published previously for EPEC and VTEC strains cultured in DMEM or MEM (Ebel *et al.*, 1996; Kenny & Finlay, 1995; Kenny *et al.*, 1997; Yoh *et al.*, 2003), although minor variations in the apparent molecular weights of the secreted proteins were observed between strains. Consequently, the identity of the prominent secreted protein bands could be predicted. The dominant protein of approximately 33 kDa is likely to be a doublet of proteins, consisting of predominantly EspB but also containing EspD. Occasionally, the two proteins were more clearly resolved with EspB and EspD having molecular weights of approximately 33 kDa and 34 kDa, respectively. The protein of approximately 22 kDa was assumed to be EspA and this was confirmed by Western blotting. The results of EspA Western blotting on the secreted protein samples are shown below the corresponding secretion profile (Figure 4.3B).

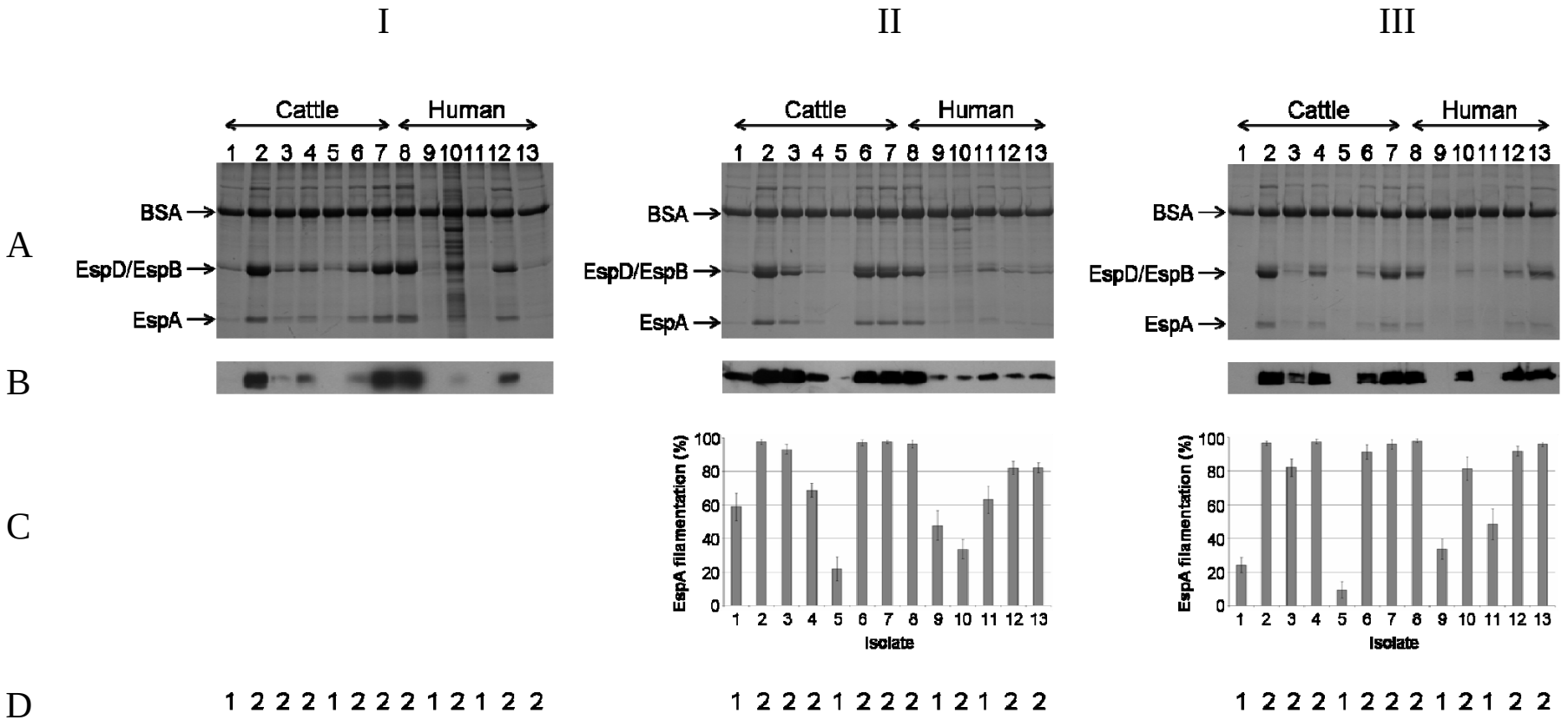

I
II
III
A
B
C
D
Cattle
Human
1 2 3 4 5 6 7 8 9 10 11 12 13
BSA
EspD/EspB
EspA
EspA filamentation (%)
0 20 40 60 80 100
Isolate
1 2 2 2 1 2 2 2 1 2 1 2 2

Figure 4.3. Analysis of LEE-encoded protein secretion by VTEC O26 isolates. Each isolate was cultured on three separate occasions and the results obtained in each instance are shown in three separate columns (columns I, II and III). (A) Secreted protein profiles from VTEC O26 isolates, ZAP1078_B2 (lane 1), ZAP1086_B10 (lane 2), ZAP1094_B18 (lane 3), ZAP1100_B24 (lane 4), ZAP1105_B29 (lane 5), ZAP1106_B30 (lane 6), ZAP1109_B33 (lane 7), ZAP1122_G14 (HUS, lane 8), ZAP1126_G18 (HUS, lane 9), ZAP1129_G21 (HUS, lane 10), ZAP1130_G22 (HUS, lane 11), ZAP1152_S35 (diarrhoea, lane 12) and ZAP1153_S36 (bloody diarrhoea, lane 13). Bacteria were cultured in DMEM-HEPES to an OD_{600} of 0.8 and supernatant proteins were TCA precipitated, separated by SDS-PAGE and visualised by staining with Colloidal blue. The positions of the co-precipitant BSA (66 kDa) and the secreted proteins EspD/EspB (~ 33 kDa) and EspA (~ 22 kDa) are indicated. (B) Western blot detection of EspA in the secreted protein samples shown in Figure 4.3A. (C) Percentage of bacteria expressing EspA filaments. Bacteria were cultured as described for secreted protein precipitation and aliquots of bacterial culture were stained for EspA filaments with EspA antibody. Proportions of bacteria expressing EspA filaments were determined by immunofluorescence microscopy as described in materials and methods. Error bars indicate standard deviations. (D) Allele number for the nucleotide sequence of the region upstream of *LEE1* in each of the isolates. Allele numbers were determined as described in section 3.2.2.

There were clear differences in the levels of protein secretion between VTEC O26 isolates cultured in DMEM. Moreover, there was noticeable heterogeneity in the levels of secretion by many of the isolates when they were cultured on different occasions. For example, while isolate ZAP1078_B2 secreted very low levels of LEE-encoded proteins, with no EspA detected by Western blotting, on two occasions (Figure 4.3A and B, columns I and III, lane 1); in one instance, Western blotting identified reasonable levels of EspA secretion (Figure 4.3B, column II, lane 1). Isolates ZAP1086_B10, ZAP1109_B33 and ZAP1122_G14 (Figure 4.3A and B, columns I, II and III, lanes 2, 7 and 8, respectively) appeared to consistently secrete high levels of LEE-encoded proteins while isolate ZAP1105_B29 (Figure 4.3A and B, columns I, II and III, lane 5) seemed to uniformly secrete low levels of protein.

The six human isolates examined harboured the genes for both Vtx1 and Vtx2 and the majority (four) were associated with HUS. However, the two isolates from cases of infection in Scotland were associated with milder disease – one was isolated from a patient with bloody diarrhoea and the second from a patient with simple diarrhoea. The results from these six human isolates suggest that there does not appear to be an association between the level of LEE protein secretion and the severity of disease. While one of the four isolates associated with HUS (ZAP1122_G14; Figure 4.3A and B, lane 8) consistently secreted high levels of protein, the remaining three isolates (ZAP1126_G18, ZAP1129_G21 and ZAP1130_G22; Figure 4.3A and B, lanes 9, 10 and 11, respectively) generally secreted much lower quantities of LEE-encoded protein. However, ZAP1129_G21 appeared more heterogeneous in its level of LEE protein secretion. Isolates ZAP1152_S35 and ZAP1153_S36 (Figure 4.3A and B, lanes 12 and 13, respectively) recovered from patients with diarrhoea and bloody diarrhoea, respectively, secreted variable levels of protein. The level of secretion by these Scottish isolates was comparable to or greater than that by the low-secreting HUS-associated isolates.

Comparison of the results from the bovine and human isolates examined suggests that the cattle isolates generally secrete higher levels of LEE-encoded protein than the majority of the human isolates. However, human isolate ZAP1122_G14 (Figure

4.3A and B, lane 8) secretes protein at levels comparable to the high-secreting bovine isolates and some cattle isolates secrete low levels of LEE effector proteins.

While twelve of the thirteen isolates had a similar secreted protein profile, albeit with variable levels of protein secretion, isolate ZAP1129_G21 had a different profile (Figure 4.3A, lane 10). The supernatants of isolate ZAP1129_G21 contained additional proteins which were clearly visible following SDS-PAGE and Colloidal blue staining. Although this isolate may genuinely secrete additional proteins, it is also possible that during culture of this isolate in DMEM a proportion of bacteria were lysed.

4.2.2 EspA filamentation

There is thus marked heterogeneity in the level of EspA, EspB and EspD secretion by VTEC O26 isolates when they are cultured in DMEM. To investigate whether this heterogeneity correlated with different proportions of the bacterial population expressing EspA filaments, the percentage of bacteria expressing EspA filaments was determined. An aliquot of bacterial culture (OD_{600} of 0.8) was fixed in PFA before the culture was centrifuged for protein precipitation. Fixed bacteria were then immunostained for surface EspA filaments and, following immunofluorescence microscopy, the percentage of bacteria expressing filaments was calculated. The proportion of the bacterial population expressing EspA filaments was determined on two occasions for each isolate investigated and the results are shown below the corresponding secreted protein profile and EspA western blot (Figure 4.3C). A representative phase contrast and fluorescence micrograph for each isolate is shown in Figure 4.4.

There was a correlation between the proportion of the bacterial population expressing EspA filaments and the levels of EspA, EspB and EspD secretion by VTEC O26 isolates. High-secreting strains had a higher percentage of EspA-positive bacteria than did low-secreting strains. High-secreting strains produced populations that had more than 90% of the bacteria expressing EspA filaments on their surface whereas low-secreting strains had less than 50%.

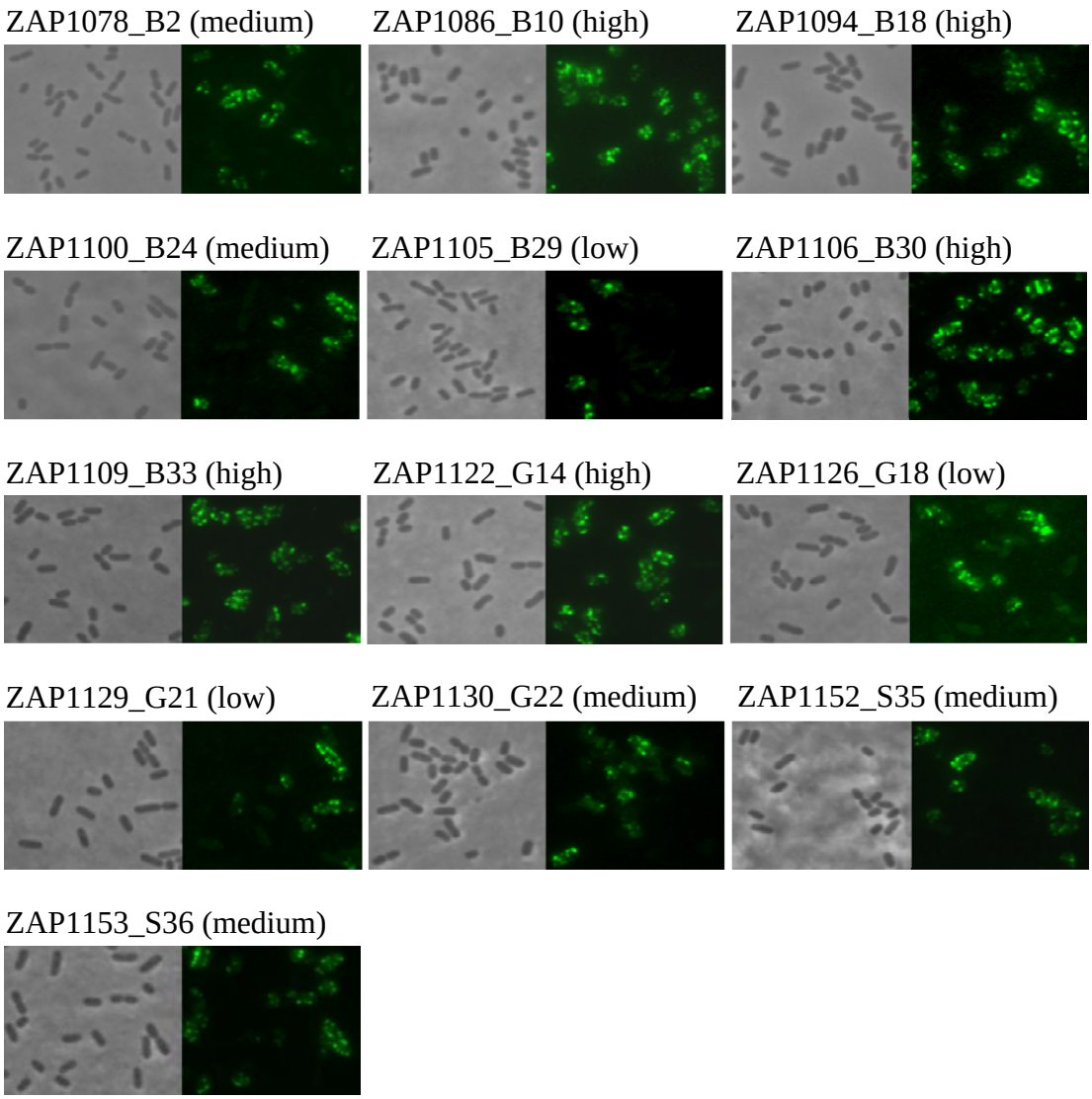


Figure 4.4. Detection of EspA filaments by immunofluorescence microscopy. Phase contrast (left panel) and fluorescence (right panel) micrographs are shown for the indicated VTEC O26 isolates stained for EspA filaments with EspA antibody. Bacteria were cultured in DMEM-HEPES to an OD_{600} of 0.8. The images presented are from the samples shown in Figure 4.3, column II and the status of the isolate for its level of LEE-protein secretion on this occasion is indicated in parenthesis. Slides were examined using an x100 objective and images were captured with Leica software.

Immunofluorescence microscopy revealed that even isolates which had no detectable levels of EspA in their supernatants were expressing EspA filaments on their surface. These isolates produced populations containing 10 to 48% EspA-positive bacteria (Figure 4.3, column III, lanes 1, 5, 9 and 11). Analysis of the fluorescence micrographs did not reveal any noticeable differences in the level of EspA filamentation on an individual EspA-positive bacterium between high- and low-secreting strains (Figure 4.4).

4.2.3 Nucleotide sequence of the promoter for *LEE1*

The obvious heterogeneity in the level of LEE-protein secretion by VTEC O26 isolates prompted investigation of the nucleotide sequence of the *LEE1* promoter possessed by each of the isolates examined. The region upstream of *LEE1*, which is likely to contain the *LEE1* promoter, was sequenced to supplement the MLST analysis of *E. coli* O26 isolates (section 3.2.2) and the allele number for this locus for each of the isolates is detailed in Figure 4.3D. There appears to be an association between the nucleotide sequence for the region upstream of *LEE1* and the level of LEE-protein secretion. Those isolates which possess allele 1 for the region upstream of *LEE1* were typically low-secreting strains whereas those which possess allele 2 were generally medium- to high-secreting strains. This association can be seen most clearly in Figure 4.3, column III.

4.2.4 Summary of LEE protein secretion results

There is considerable variation in the level of LEE-encoded protein secretion by VTEC O26 isolates when they are cultured in DMEM and the level of secretion correlated with the proportion of the bacterial population expressing EspA filaments. Among the VTEC O26 isolates analysed, it appeared that those isolates which possess allele 1 for the region upstream of *LEE1* were typically low-secreting strains whereas those which possess allele 2 were generally medium- to high-secreting strains. There did not appear to be an association between the level of LEE-encoded protein secretion and the ability to cause more serious disease. However, in general, the Scottish cattle isolates appeared to secrete higher levels of protein than the human isolates.

4.3 Adherence assays

While type III secretion-based intimate attachment and A/E lesion formation are likely to be important for intestinal colonisation, adherence of the bacterium mediated by surface-expressed factors such as fimbriae may also contribute. It was investigated whether VTEC O26 isolates associated with more serious disease could adhere to human intestinal epithelial cells at higher levels than those associated with mild disease and whether Scottish cattle isolates could adhere to human intestinal epithelial cells.

Thirteen VTEC O26 isolates, seven bovine and six human, positive for both *vtx*$_1$ and *vtx*$_2$ genes were cultured on CFA agar at 37ºC and tested for their capacity to adhere to Caco-2 cells within 2 h. CFA medium has been used previously to promote fimbrial expression (Evans *et al.*, 1977). The average number of bacteria attached to Caco-2 cells per field of vision for each VTEC O26 isolate is shown in Figure 4.5. One-way analysis of variance analysis of the number of bacteria attached to Caco-2 cells per field for the thirteen VTEC O26 isolates demonstrated significant variation ($P < 0.001$). Individual differences were investigated using Tukey's test for multiple comparisons, with a family error rate of 1%. This analysis divided the isolates into three groups based on their levels of adherence to Caco-2 cells. Isolates ZAP1078_B2, ZAP1130_G22 and ZAP1153_S36 adhered to Caco-2 cells at significantly higher levels than the remaining VTEC O26 isolates. Isolates ZAP1105_B29, ZAP1126_G18, ZAP1129_G21 and ZAP1152_S35 adhered to Caco-2 cells at moderate levels, while isolates ZAP1086_B10, ZAP1094_B18, ZAP1100_B24, ZAP1106_B30, ZAP1109_B33 and ZAP1122_G14 adhered to Caco-2 cells at low levels.

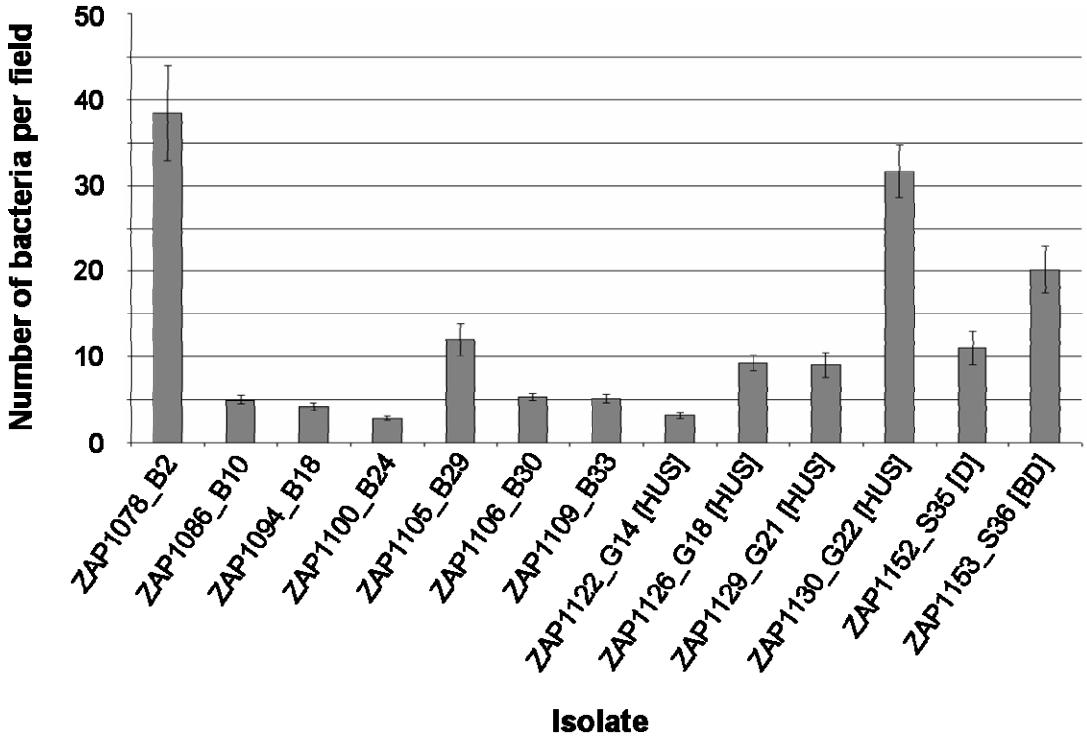


Figure 4.5. Adherence of VTEC O26 isolates to Caco-2 cells. The indicated isolates were cultured on CFA agar at 37ºC and adherence assays were performed at 37ºC for 2 h. Microscopy was used to determine the number of bacteria per field attached to Caco-2 cells for 30 fields. Adherence assays for each isolate were performed on at least three separate occasions. The bars represent the average number of attached bacteria per field and error bars indicate standard errors of the means. The clinical disease associated with each human isolate is indicated in parenthesis after the isolate number: D, diarrhoea; BD, bloody diarrhoea; HUS, haemolytic uraemic syndrome.

VTEC O26 isolates recovered from clinical cases of human infection appear to adhere to Caco-2 cells at higher levels than isolates recovered from Scottish cattle. The majority (5/6, 83%) of human isolates adhered to Caco-2 cells at moderate to high levels whereas most (5/7, 71%) of the cattle isolates only adhered at low levels (Figure 4.5). There does not appear to be an association between the level of adherence by human isolates and the severity of disease. While one of the four isolates associated with HUS (ZAP1122_G14) adhered to Caco-2 cells at low levels, the remaining three isolates adhered at higher levels. Isolates ZAP1152_S35 and ZAP1153_S36, recovered from patients with diarrhoea and bloody diarrhoea, adhered to Caco-2 cells at moderate and high levels, respectively (Figure 4.5).

Interestingly, the VTEC O26 isolates which typically secreted low levels of LEE-encoded proteins [namely, ZAP1078_B2, ZAP1105_B29, ZAP1126_G18 and ZAP1130_G22 (Figure 4.3)] adhered to Caco-2 cells at much higher levels than those isolates which consistently secreted high levels of LEE-encoded proteins [namely, ZAP1086_B10, ZAP1109_B33 and ZAP1122_G14 (Figure 4.3)].

Microscopy revealed that, in addition to many individual bacteria, isolates ZAP1078_B2 and ZAP1130_G22 also had small aggregates of bacteria attached to Caco-2 cells. Thin aggregative fimbriae called curli are an established adhesin and, therefore, the ability of VTEC O26 to express curli was investigated. Congo red (CR) binding is a well-established method to demonstrate surface curli expression by bacteria (Barnhart & Chapman, 2006; Hammar *et al.*, 1995; Uhlich *et al.*, 2001). Curliated bacteria are able to bind CR dye and stain red. The VTEC O26 isolates were cultured on CFA-CR indicator plates at 37ºC and while the majority of isolates were negative, isolates ZAP1078_B2 and ZAP1130_G22 were positive. This data indicates that isolates ZAP1078_B2 and ZAP1130_G22 express curli at 37ºC on CFA agar.

4.3.1 Summary of adherence assay results

Adherence assays were performed for a standard 2 h at 37°C on Caco-2 cells which had been incubated at 37°C in 5% CO_2 for 48 h prior to the experiment. There is significant variation in the capacity of VTEC O26 isolates to adhere to Caco-2 cells following culture on CFA agar at 37°C. Human VTEC O26 isolates appeared to adhere to Caco-2 cells at higher levels than the cattle isolates, but there did not appear to be an association between the level of adherence by human isolates and the severity of disease. The VTEC O26 isolates which typically secreted low levels of LEE proteins adhered to Caco-2 cells at moderate to high levels whereas those which secreted high levels of LEE proteins only adhered at low levels. Two of the isolates which adhered to Caco-2 cells at higher levels stained red on CFA-CR indicator plates at 37°C, indicating surface curli expression.

4.4 Discussion

The presence of a *vtx* gene, particularly vtx_2, and the LEE pathogenicity island in an infecting strain is considered an important indicator of human pathogenic potential. However, knowledge of whether these genes are, in fact, functional and an understanding of their level of expression in certain strains is also highly relevant. This chapter described certain phenotypic characteristics of VTEC O26 isolates by investigating levels of verocytotoxin production and LEE-protein secretion. It also considered the ability of VTEC O26 to adhere to Caco-2 cells.

Vtx production is the principal virulence attribute of *E. coli* strains and appears to be an important factor in the development of serious human disease. It is possible that progression to more severe disease could be accounted for by increased Vtx production. The production of Vtx by VTEC O26 isolates was investigated using an accepted technique, the Vero cell cytotoxicity assay, where the Vtx titre is dependent upon the level of toxin secreted and the cytotoxic activity of the toxin towards Vero cells.

A key observation following the Vero cell cytotoxicity assays was that culture filtrates from non-induced VTEC O26 isolates harbouring vtx_2 alone were not cytotoxic to Vero cells. Filtrates from VTEC O26 isolates containing vtx_1, either alone or in combination with vtx_2, were verocytotoxic. Furthermore, while mitomycin C induction increased the Vtx titres in the culture filtrates of the majority of vtx_2-positive isolates to levels which were cytotoxic to Vero cells, these filtrates had a much lower cytotoxic activity on Vero cells compared to those from mitomycin C-induced VTEC O26 isolates that carried vtx_1. Since Vtx2-producing VTEC appear to have a greater pathogenicity in clinical situations, it was surprising to find that culture filtrates supposedly containing Vtx2 were not as cytotoxic to Vero cells as those containing Vtx1. Although previous research has shown that VTEC strains, including those belonging to serogroup O26, can lose their *vtx* genes during subculture (Bielaszewska *et al.*, 2007; Karch *et al.*, 1992), none of the VTEC O26 isolates examined in this study has lost their *vtx* gene(s) through culture. Therefore, the much lower cytotoxic activity on Vero cells by culture filtrates from isolates

harbouring *vtx*$_2$ alone cannot be explained by the loss of the *vtx*$_2$ gene. However, there are other possible explanations for the lower cytotoxic activity on Vero cells by culture filtrates from isolates carrying *vtx*$_2$ alone.

Firstly, it is possible that the Vero cells are not as sensitive to Vtx2 as they are to Vtx1. An earlier study by Strockbine *et al.* (1986) compared the biological activities of Vtx1 and Vtx2 by analysing the cytotoxin in the cell lysates of *E. coli* K-12 lysogenised with phage 933J (Vtx1) and phage 933W (Vtx2) from *E. coli* O157:H7 EDL933. While both toxins were cytotoxic to Vero cells, Vtx2, per unit of protein, was less cytotoxic than Vtx1. Moreover, research by Karmali's group (Head *et al.*, 1988) found that "on a weight basis, Vtx2 was 1,000-fold less active on Vero cells than Vtx1" (Karmali, 1989); although the Vtx2 prepared in this instance was probably not pure, but instead consisted of the two comparable but not identical toxins, Vtx2 and Vtx2c (Schmitt *et al.*, 1991). However, the Vero cell cytotoxicity assay is the conventional technique used to detect the production of Vtx by VTEC and has been used routinely to confirm the presence of both Vtx1 and Vtx2 in culture and faecal filtrates (Bielaszewska *et al.*, 2007; Karmali *et al.*, 1999; Mellmann *et al.*, 2005). Previous research has yielded positive Vero cell cytotoxicity assay results for Vtx2 in faecal filtrates (Mellmann *et al.*, 2005) and culture filtrates (Bielaszewska *et al.*, 2006; Bielaszewska *et al.*, 2007; Mellmann *et al.*, 2008). Moreover, a study comparing the performance of a commercial latex agglutination assay with that of the Vero cell cytotoxicity assay for the detection of Vtx1 and Vtx2 in *E. coli* culture filtrates found that all Vtx2-producing strains analysed gave a positive result in the Vero cell assay and made no mention of any observation that Vtx2 was less active than Vtx1 on Vero cells (Karmali *et al.*, 1999). Differences in the cytotoxicity of Vtx2-producing strains to Vero cells observed among laboratories could be due to variations in expression of the *vtx*$_2$ gene under different experimental growth conditions and/or variations in the sensitivities of different Vero cell lines.

Secondly, the culture filtrates from isolates harbouring *vtx*$_2$ alone may have had a much lower cytotoxic activity on Vero cells than those from isolates containing *vtx*$_1$ because they contained lesser amounts of Vtx. Perhaps the non-induced *vtx*$_2$-positive

isolates produced Vtx2 in quantities that were below the level of detection by the Vero cell assay. It is possible that only low levels of Vtx2 are produced because Vtx2 is trafficked to target organs more efficiently than Vtx1 and, once there, Vtx2 may elicit a more severe cytotoxic response. Previous research has shown that Vtx1 binds to the surface of intestinal epithelial cells *in vitro* with a higher affinity than Vtx2 (Hurley *et al.*, 1999) and may, therefore, be 'held up' in the intestine *in vivo*. Although more Vtx1 than Vtx2 translocates across intestinal epithelial cells *in vitro* (Hurley *et al.*, 1999), the intestinal capillary network is believed to be the first endothelial cell target to be encountered following Vtx translocation across the intestinal epithelial cell layer (Jacewicz *et al.*, 1999), and the binding affinity of Vtx1 to human intestinal microvascular endothelial cells was much greater than that of Vtx2 *in vitro* (Jacewicz *et al.*, 1999). In addition, it has been reported that Vtx2 was more toxic to human intestinal microvascular endothelial cells than Vtx1 (Jacewicz *et al.*, 1999). The decreased binding affinity and increased toxicity for intestinal endothelial cells of Vtx2 compared to Vtx1 may facilitate the access of Vtx2 to the systemic circulation to reach the kidney. Here, Vtx will act on renal endothelial cells and Vtx2 has been demonstrated to be 1,000 times more toxic for human renal microvascular endothelial cells than is Vtx1 (Louise & Obrig, 1995).

To gain further information about whether the lower cytotoxic activity on Vero cells of the culture filtrates from isolates harbouring vtx_2 alone compared to those from isolates carrying vtx_1 could be due to (1) reduced sensitivity of the Vero cells to Vtx2 or (2) lower levels of Vtx2 production, it would be necessary to perform additional experiments to characterise Vtx production. This may include: confirming Vtx1 and/or Vtx2 production and release using a commercial latex agglutination assay (Karmali *et al.*, 1999) or an immuno-PCR assay (Zhang *et al.*, 2008); determining Vtx concentrations in the culture filtrates by ELISA (Ritchie *et al.*, 2003); or analysing levels of *vtx* gene expression by quantitative PCR (Q-PCR) (Zhang *et al.*, 2005).

Thirdly, it is also possible that the reduced cytotoxicity to Vero cells of culture filtrates from isolates carrying vtx_2 alone was a consequence of production of a Vtx2

variant which has low cytotoxic activity on Vero cells. Previous research has shown Vtx2c is less active than Vtx2 on Vero cells (Lindgren *et al.*, 1994). Therefore, it is necessary to determine the *vtx*$_2$ subgroup harboured by the isolates positive for *vtx*$_2$ alone to ascertain whether a Vtx2 variant may be responsible for the reduced cytotoxic activity on Vero cells.

Interestingly, the ability of mitomycin C to increase the Vtx titres in the culture filtrates from five of six of the isolates positive for *vtx*$_2$ alone indicates that, in these isolates, the *vtx*$_2$ gene is functional. However, since Vtx titres by ZAP1128_G20 were not increased in response to mitomycin C it is possible that the *vtx*$_2$ gene of this strain is not intact, not phage borne or is located on a defective phage. It would be necessary to perform sequence analysis of the *vtx*$_2$ gene from ZAP1128_G20 to investigate whether a mutation or truncation of *vtx*$_2$ may account for the lack of verocytotoxicity of the culture filtrate from this isolate.

The Vero cell cytotoxicity assay also demonstrated that the culture filtrate from the non-induced *vtx*$_1$-positive isolate ZAP1146_I29 had a higher Vtx titre than the culture filtrates from the other isolates harbouring *vtx*$_1$. Notably, the greater cytotoxicity of this isolate to Vero cells correlated with the progression to HUS. Although the production of Vtx1 alone is not generally associated with HUS, this increased cytotoxicity may contribute to the enhanced pathogenicity of this isolate. It is possible that the greater cytotoxicity to Vero cells is due to the production of greater quantities of Vtx1. This could be the consequence of either, increased expression of a single *vtx*$_1$ gene or the presence and expression of multiple copies of *vtx*$_1$ genes. Although multiple copies of *vtx*$_2$ genes have been reported in VTEC strains (Bielaszewska *et al.*, 2006; Schmitt *et al.*, 1991), Schmitt *et al*. (1991) did not identify multiple copies of the *vtx*$_1$ gene in any of the *E. coli* isolates examined. To investigate these possibilities further it would be necessary to determine levels of Vtx production more precisely using the techniques mentioned previously, and ascertain the number of copies of *vtx*$_1$ harboured by the VTEC O26 isolates by restriction enzyme digestion of genomic DNA and subsequent Southern blotting using a *vtx*$_1$ probe (Bielaszewska *et al.*, 2006; Schmitt *et al.*, 1991).

An alternative explanation for the greater cytotoxicity of isolate ZAP1146_I29 to Vero cells is the production of a more active toxin variant. Indeed, Vtx2 variants have exhibited different toxicities to Vero cells (Lindgren *et al.*, 1994). However, this is unlikely since Vtx1 is generally more conserved than Vtx2 and isolates harbouring the *vtx*$_1$ variant, *vtx*$_{1c}$, had comparable Vero cell cytotoxicity titres to isolates carrying *vtx*$_1$ (Zhang *et al.*, 2002). In addition, only *vtx*$_1$ has been detected in VTEC O26 isolates previously (Jenkins *et al.*, 2003; Zhang *et al.*, 2002). However, to investigate the possibility that the high Vtx titre of ZAP1146_I29 is due to a more cytotoxic variant, the *vtx*$_1$ gene(s) carried by the VTEC O26 isolates should be sequenced.

Overall, there was no evidence of an association between Vtx titre and the risk of serious complications following infection with *E. coli* O26 strains. With the exception of isolate ZAP1146_I29, which was recovered from a case of HUS, there were no major differences in Vtx titres between non-induced isolates, which harboured *vtx*$_1$ alone, from cases of diarrhoea and HUS. In addition, there did not appear to be a correlation between the level of increase in Vtx titres by the *vtx*$_1$-positive isolates following mitomycin C treatment and the severity of disease. Furthermore, the Vtx titres of non-induced isolates, carrying both *vtx*$_1$ and *vtx*$_2$, from diarrhoea, bloody diarrhoea and HUS infections were similar. The lack of association between levels of Vtx production and clinical outcomes of infection is in accordance with two previous studies (Cornick *et al.*, 2002; Zhang *et al.*, 2005) but does not agree with other research, which demonstrated that Vtx quantities produced by infecting VTEC strains correlated with the clinical outcomes of infection (Eklund *et al.*, 2002; Muniesa *et al.*, 2003). However, it is appreciated that the conditions exposed to *in vivo* may result in expression differences not observed *in vitro*.

While increased Vtx production may not account for the greater pathogenicity of certain VTEC O26 isolates, it is possible that these isolates may colonise the human intestine in higher numbers, resulting in increased exposure to toxin and progression to more serious disease. The ability of *E. coli* strains to form A/E lesions promotes colonisation of the intestinal mucosa and the production and secretion of LEE-

encoded proteins is necessary for the formation of A/E lesions. Therefore, the ability of VTEC O26 to secrete LEE-encoded proteins was investigated. The VTEC O26 isolates analysed harboured the LEE pathogenicity island and carried the genes for both Vtx1 and Vtx2. LEE-secreted protein production by each isolate was investigated by analysing the supernatant proteins following culture in DMEM medium, which has been shown to be permissive for LEE-protein secretion (Ebel *et al.*, 1996; Kenny & Finlay, 1995). Supernatant proteins were TCA precipitated, separated by SDS-PAGE and visualised by Colloidal blue staining and EspA Western blotting. Clear differences were detected in the level of secretion of LEE-encoded proteins between VTEC O26 isolates when cultured in DMEM. Such apparent variation in LEE-protein secretion levels has been observed previously among *E. coli* O157:H7 strains cultured in MEM (Roe *et al.*, 2003; unpublished results). The study by Roe *et al.* (2003) demonstrated that the level of LEE-protein secretion by *E. coli* O157:H7 strains was associated with the proportion of the bacterial population expressing EspA filaments. Consequently, the presence of EspA filaments on the surface of the VTEC O26 isolates following culture in DMEM was determined by immunofluorescent staining with an EspA antibody, and the percentage of bacteria expressing filaments was calculated.

The results from this analysis indicated that EspA secretion by VTEC O26 is heterogeneous. Only a proportion of the bacterial population from each isolate investigated had EspA filaments and were secreting proteins; the remaining bacteria lacked filaments and presumably were not secreting. The extent of EspA filamentation in the bacterial population varied both between isolates and when the same isolate was cultured on different occasions. The proportion of the bacterial population expressing filaments correlated with the observed differences in levels of LEE-encoded protein secretion – high-secreting strains expressed filaments on a greater proportion of the population than did low-secreting strains. Heterogeneous or limited, tightly controlled expression of EspA may reduce the likelihood of an immune response to this antigen, as suggested previously (Roe *et al.*, 2003).

The molecular basis of the heterogeneity in LEE-protein secretion levels between VTEC O26 isolates was considered. The region upstream of *LEE1*, which is likely to contain the *LEE1* promoter, was sequenced to supplement the MLST analysis of *E. coli* O26 isolates (section 3.2.2), and there appeared to be an association between the nucleotide sequence at this locus and the level of LEE-protein secretion by VTEC O26 isolates harbouring both vtx_1 and vtx_2 genes. Those isolates which possessed allele 1 for the region upstream of *LEE1* were typically low-secreting strains whereas those which possessed allele 2 were generally medium- to high-secreting strains. In the 644 bp of sequence data determined, alleles 1 and 2 differ by a single nucleotide and this base pair difference lies in an area where regulators are known to act in *E. coli* O157:H7 [between base pairs -218 and -123 to the P2 *LEE1* promoter (Figure 3.3; see Appendix 3)] and is in the region where IHF is known to bind in *E. coli* O157:H7 (Figure 3.3). Thus, it is possible that such sequence variation may result in differences in the potential of regulators to bind in this region. The effect of sequence differences in the *LEE1* promoter on protein secretion by *E. coli* O26 merits further investigation. Since analysis of secreted protein levels was only performed on a limited number of isolates, all of which had the same *vtx* genotype, it would be necessary to examine a larger collection of isolates harbouring these different nucleotide sequences to see if this relationship holds true. In the limited number of isolates examined there did not appear to be an association between the level of LEE-protein secretion and the ability to cause more serious clinical disease.

In addition to type III secretion-based intimate attachment, intestinal colonisation may be facilitated by adherence mediated by surface-expressed appendages such as fimbriae. Therefore, the capacity of VTEC O26 isolates to adhere to a human intestinal epithelial cell line within 2 h was tested. Culture of the isolates on CFA agar at 37ºC resulted in significant variation in the number of bacteria adhering to Caco-2 cells. However, there was no apparent association between the level of adherence and severity of disease. It is interesting to note that the VTEC O26 isolates which generally secreted low levels of LEE-encoded proteins adhered to Caco-2 cells at higher levels than those isolates which consistently secreted high levels of LEE-encoded proteins. It is possible that the expression of factors on the

bacterial cell surface is coordinated to reduce the likelihood of redundant adherence mechanisms being simultaneously produced. Those isolates which exhibit low levels of LEE-protein secretion have a lower proportion of bacterial cells secreting the translocon proteins and this may allow other adhesins to function without interference from EspA filaments. It is possible that these adhesins drive the initial adherence of VTEC O26 to human intestinal epithelium which in turn leads to the production of the translocon apparatus.

Two of the VTEC O26 isolates that adhered to Caco-2 cells at high levels bound congo red when cultured on CFA plates at 37ºC. This indicates that these isolates express curli under such conditions, although further analysis, such as immunostaining with a curli antibody, is required for confirmation. Curli are an established adhesin and possibly contribute to the increased adherence observed for these two VTEC O26 isolates. To establish the contribution of curli in their adherence it would be necessary to create a curli mutant in each of the isolates and then evaluate the adherence of the mutant compared to the parent strain.

Since one of the VTEC O26 isolates that adhered to Caco-2 cells at high levels and all four of the isolates that adhered at moderate levels did not bind congo red when cultured on CFA agar at 37ºC indicates that these isolates do not express curli under these conditions. It is likely that adherence by these isolates is mediated by alternative surface factor(s). Indeed, at least sixteen putative fimbrial operons have been identified in *E. coli* O157:H7 (Hayashi *et al.*, 2001; Low *et al.*, 2006; Perna *et al.*, 2001). Microarray analysis of gene expression by VTEC O26 isolates following culture on CFA agar at 37ºC may indicate adhesins which are potentially contributing to adherence under these conditions.

There did not appear to be an association between (1) Vtx titre, (2) level of LEE-protein secretion, or (3) level of adherence to Caco-2 cells and the ability to cause more serious human disease. However, it should be remembered that host factors, including age, clinical history, or treatment issues such as the administration of antibiotics and/or anti-motility agents may also be important in determining the

clinical outcome of infection. VTEC O26 isolates recovered from patients in continental Europe appeared to produce higher levels of Vtx than their Scottish counterparts, although further work is needed to confirm this. Although there were no clear differences in the levels of LEE-protein secretion between VTEC O26 isolates recovered from patients in Scotland and continental Europe, only a limited number of isolates (those which carried the genes for both Vtx1 and Vtx2) were examined. If the *LEE1* promoter sequence proves to be indicative of LEE-protein secretion levels, then the fact that the majority of VTEC O26 isolates recovered in continental Europe carried allele 1 whereas all of the VTEC O26 isolates recovered from patients in Scotland possessed allele 2 (Table 3.10) suggests that the majority of VTEC O26 from continental Europe should be lower secretors than those from Scotland. The contribution this phenotype may have on the capacity to cause more serious human disease is unknown. Perhaps these low-secreting isolates have a more tightly regulated T3SS, which limits potential immune exposure, but can be induced at their desired site of colonisation once permissive environmental factors are encountered. However, recent work has demonstrated that O-islands carry effector proteins, regulators and surface adhesins that can alter the regulation of the T3SS (Tobe *et al*., 2006; ZAP group unpublished results). Therefore, the level of LEE-encoded protein secretion may be a marker of the O-island-phage repertoire of the strain and the low-secretion phenotype is simply a marker of the presence or absence of DNA which is necessary to allow these strains to cause more serious disease.

Analysis of VTEC O26 isolates recovered from Scottish cattle in Vero cell cytotoxicity assays found that the culture filtrates from non-induced isolates carrying either vtx_1 alone or vtx_1 and vtx_2 in combination had comparable Vtx titres to those from non-induced VTEC O26 isolates recovered from patients in Scotland, but lower Vtx titres to those from non-induced VTEC O26 isolates recovered from clinical cases in continental Europe, with the same *vtx* genotype. This is in agreement with the findings of Ritchie *et al*. (2003), who demonstrated that basal levels of Vtx1 or Vtx2 production by HUS-associated isolates was significantly greater than that by bovine isolates. However, further analysis of the vtx_1-positive isolates in my study found that mitomycin C treatment generally increased Vtx titres more in the cattle

isolates than in the human isolates and, as a result, there was no significant difference in the Vtx titres between the cattle and human isolates following mitomycin C induction. Since the human body contains other prophage-inducing agents, such as H_2O_2 released by neutrophils (Wagner & Waldor, 2002), cattle isolates might produce comparable quantities of Vtx to disease-associated isolates in the human host if they successfully colonise human beings.

In the small number of VTEC O26 isolates investigated, it appeared that those recovered from Scottish cattle generally secreted higher levels of LEE-encoded proteins and adhered to Caco-2 cells at lower levels than the majority of isolates from cases of human infection. On the basis of these phenotypic results it appears that the majority of Scottish cattle isolates have different characteristics to those causing human disease, and this could explain the low incidence of *E. coli* O26 infections in Scotland. However, the Scottish cattle isolate which, for the genotypic characteristics investigated, was indistinguishable from the isolate recovered from a patient with HUS remains a serious concern. This isolate consistently secreted low levels of LEE-encoded proteins and adhered to Caco-2 cells at moderate levels and was thus comparable to many of the human isolates associated with severe disease. Moreover, a further isolate recovered from a case of HUS in continental Europe carried *vtx*$_1$, *vtx*$_2$, LEE, *hlyA* and allele 2 for the region upstream of *LEE1*, and consistently secreted high levels of LEE-encoded proteins and adhered to Caco-2 cells at low levels. With the exception of the carriage of *tccP2*, this isolate was comparable to a number of VTEC O26 strains residing in Scottish cattle.

4.5 Section I discussion

VTEC O26 strains have emerged as significant human pathogens in continental Europe, often causing severe diarrhoea that can progress to life-threatening HUS. In contrast, human infections associated with *E. coli* O26 are uncommon in Scotland and are generally associated with simple diarrhoea. One of the aims of this study was to characterise *E. coli* O26 isolates from these different locations to identify bacterial factors which allow these pathogens to cause more serious disease.

Nucleotide sequencing of housekeeping genes found very little genetic variation in the genomic 'backbone' among *E. coli* O26 isolates recovered from patients in Scotland and continental Europe. *E. coli* O26 has considerable genomic variation which is presumed, therefore, to occur as a result of the insertion and deletion of horizontally acquired genomic O-islands and bacteriophages. Thus, it is probable that the difference in human pathogenic potential between these two groups of isolates is due to horizontally acquired DNA. Indeed, differences between these two groups emerged following investigation of virulence gene carriage. A number of VTEC O26 isolates recovered in continental Europe harboured the *vtx*$_2$ gene alone and the majority of European VTEC O26 carried allele 1 for the region upstream of *LEE1* and were positive for *tccP2*. In contrast, the majority of Scottish human isolates were *vtx*-negative and, among those which were *vtx*-positive, no isolate carried the *vtx*$_2$ gene alone. Furthermore, all VTEC O26 recovered from patients in Scotland carried allele 2 for the region upstream of *LEE1* and were negative for *tccP2*. Whether any of these factors are directly responsible for the increased pathogenesis observed for European O26 isolates is unknown. It is possible that the carriage of any of these factors is an indicator of a certain clonal type, which may harbour some yet-undiscovered virulence factor.

The development of an understanding of the gene repertoire of the strains studied has provided important knowledge about their virulence potential. However, virulence factor expression levels may also be essential in predicting the human pathogenic potential of an isolate. It is possible that increased production of Vtx by isolates recovered from patients in continental Europe may account for why human infections

in Europe are more severe, and perhaps low levels of LEE-protein secretion are a marker for enhanced pathogenesis. However, perhaps the ability of VTEC O26 bacteria to produce Vtx or LEE-encoded proteins is not sufficient for an organism to cause human disease without suitable additional virulence factors. It is possible that, despite the characterisation of the bacterial isolates in this study, there could be some additional, and perhaps unknown, virulence factors associated with pathogenesis. To investigate this possibility, it would be interesting to perform DNA-DNA hybridisations or high-throughput sequencing for a more thorough examination of the virulence genes and genomic islands harboured by *E. coli* O26 isolates.

In addition to the virulence factors considered in this study there are many other pathogen-related factors which may contribute to the potential to cause serious human disease and would be interesting to investigate. These include the capacity of strains to survive the harsh conditions in the gastrointestinal tract and resist the immune system and their ability to compete with other organisms in the colonic flora. It should also be remembered that the clinical outcome of infection is the end result of host, pathogen and environmental interactions. Therefore factors, other than bacterial characteristics, are also likely to contribute to disease patterns seen. These include exposure and infective doses and host susceptibility.

The second aim of this study was to investigate the potential of *E. coli* O26 strains from Scottish cattle to cause human infection. Characterisation of bovine isolates found that they were generally comparable to the Scottish human isolates. They possessed similar virulence gene profiles and produced equivalent levels of Vtx. From these results it would be feasible to suggest that bovine *E. coli* O26 are already the source of human infection in Scotland. However, as trace-back studies are unlikely, causal proof of this epidemiological link will remain lacking. The fact that no common PFGE profile has been detected between human and cattle O26 isolates recovered in Scotland (L. Allison, personal communication) is possibly the consequence of strains frequently changing their genomic ‘make-up’, for example by the loss and gain of *vtx*-converting bacteriophages as has been observed previously (Bielaszewska *et al.*, 2007; Mellmann *et al.*, 2005). If bovine *E. coli* O26 are the

source of human infection in Scotland, it would appear that they do not cause severe disease. However, it is of concern that one of the Scottish cattle isolates was very similar to isolates recovered from patients with HUS in continental Europe. This isolate carried *vtx*$_1$, *vtx*$_2$, LEE, *hlyA*, *tccP2* and allele 1 for the region upstream of *LEE1*, secreted low levels of LEE-encoded proteins and adhered to Caco-2 cells at moderate levels. It would be interesting to investigate a wider set of cattle isolates for these characteristics and surveillance for VTEC O26 isolates of this genotype is recommended. In addition, there are a number of aEPEC O26 isolates in Scottish cattle which harbour *tccP2* and allele 1. Although, in the absence of *vtx* genes, these strains are less of a threat to human health they only need to acquire the Vtx2-encoding bacteriophage and they could prove to be highly pathogenic. This event is feasible since *E. coli* O26 are a highly dynamic group of organisms that have been shown to convert from aEPEC to VTEC via the gain of Vtx-encoding phages (Bielaszewska *et al.*, 2007).

It was observed that there were differences in the level of LEE-protein secretion between cattle and human isolates, with the cattle isolates generally secreting higher levels of protein than the human isolates *in vitro*. This indicates that there are differences in the regulation of a key colonisation factor between these two groups of isolates. The impact these differences may have *in vivo* is not clear, but if they are important in the potential of a strain to transmit to the human host, there may be a reduction in the proportion of cattle isolates which could be transmitted. However, some cattle isolates were comparable to the human isolates and secreted low levels of LEE-encoded proteins. It is possible that these isolates may be able to transmit to humans and the genotypic and phenotypic characterisations suggest that *E. coli* O26 bovine isolates may then represent a threat to human health.

Although this is an important finding, there are many other variables that must exist in determining the potential of a strain to be transmitted from a bovine to a human host and then cause disease. One issue is the environmental exposure dose, with greater human exposure possibly resulting in an increased risk of infection. Elements which contribute to exposure dose are the carriage rate in cattle, bacterial

shedding levels by cattle, the ability to survive in the environment and undefined differences in animal management, husbandry or human lifestyle that could influence exposure. A comparison of the situation of *E. coli* O26 in Scottish cattle to that of *E. coli* O157:H7, where cattle isolates are known to cause human disease, is summarised in Figure 4.6. The percentage of farms on which shedding was detected was comparable for both organisms – 22.8% for *E. coli* O157:H7 (Gunn *et al*., 2007) and 23% for *E. coli* O26 (Pearce *et al*., 2006). However, of the faecal pats tested, 7.9% were positive for *E. coli* O157:H7 (Gunn *et al*., 2007) whereas only 4.6% were positive for *E. coli* O26 (Pearce *et al*., 2006). These data indicate that the carriage rate of *E. coli* O26 in Scottish cattle is lower than that of *E. coli* O157:H7 so there is presumably a reduced exposure to *E. coli* O26. It appears important to compare cattle and human *E. coli* O26 isolates in animal studies to investigate their ability to colonise the bovine host and the subsequent shedding levels and duration of colonisation. Previous studies with *E. coli* O157:H7 have indicated that cattle which are colonised at the terminal rectum are associated with high levels of faecal excretion and a longer duration of faecal shedding (Cobbold *et al.*, 2007; Lim *et al.*, 2007; Low *et al.*, 2005). It is possible that Scottish cattle are not shedding high levels of *E. coli* O26 or are only shedding for a short term, thus reducing the risk of human infection. In addition, *E. coli* O26 can be a pathogen for cattle (Pearson *et al.*, 1999; Wieler *et al.*, 1996) and this could affect shedding levels and patterns.

In addition to possible differences in the environmental load of these two pathogens, while the vast majority (99.8%) of *E. coli* O157:H7 isolates excreted by Scottish cattle harbour the virulence genes vtx_2 and *eae* (Gunn *et al*., 2007), only a limited number (10%) of bovine *E. coli* O26 isolates from Scotland possess both of these genes (Pearce *et al*., 2006) (Figure 4.6). Furthermore, a further reduced number of VTEC O26 isolates from Scottish cattle carry *tccP2* and allele 1 for the region upstream of *LEE1*, which are markers for increased potential to cause serious human disease (this study). The limited exposure of individuals in Scotland to more virulent *E. coli* O26 isolates may explain the differences in the incidence of human infection seen across Europe.

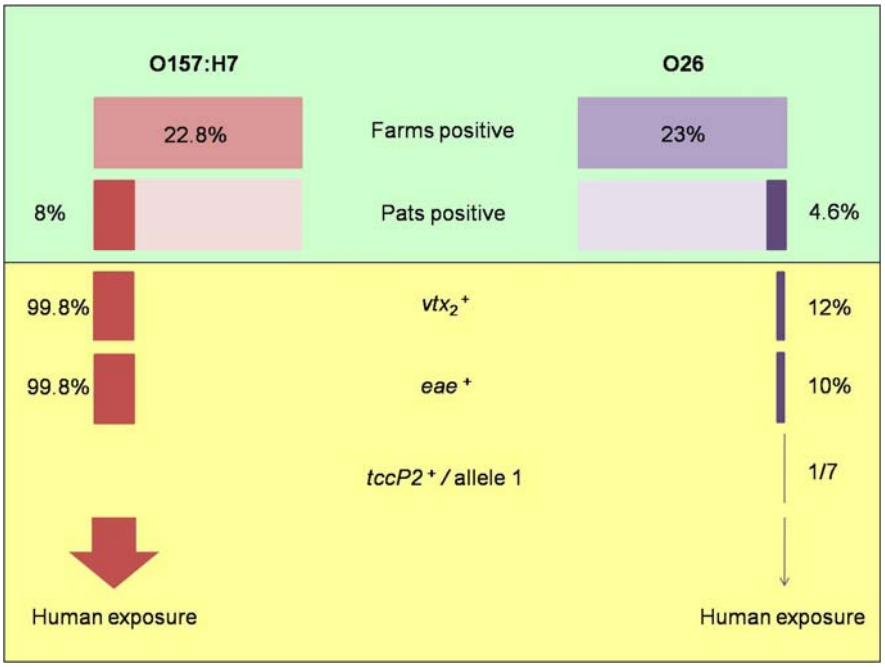


Figure 4.6. Comparison of the chain of 'filters' that may influence the potential of *E. coli* O157 and *E. coli* O26 from Scottish cattle to cause human disease. The green background corresponds to the environmental load of the two pathogens and the yellow background highlights the carriage of important virulence factors by these pathogens. The thickness of the blocks and arrows is indicative of the potential exposure dose to human beings of these two pathogens harbouring the said virulence determinants. See text for further explanation. Data obtained from this thesis, Gunn *et al*. (2007) and Pearce *et al*. (2006).

In conclusion, while the study is insufficient to form a complete risk analysis, the genotypic and phenotypic characterisations indicate that *E. coli* O26 bovine isolates from Scotland may represent a threat to human health. This should be borne in mind when seeking to manage the risk to human health in Scotland and continued surveillance and research is needed to monitor this threat.

Section II

Characterisation of sorbitol-fermenting *Escherichia coli* O157:NM isolates

VTEC strains of serogroup O157 are associated with human disease including diarrhoea, haemorrhagic colitis and HUS. While non-sorbitol-fermenting (NSF) VTEC O157:H7 strains are significant human pathogens world-wide (Mead & Griffin, 1998), sorbitol-fermenting (SF) VTEC O157:NM strains have emerged as important pathogens in continental Europe (Karch & Bielaszewska, 2001). Although SF VTEC O157:NM strains were first isolated in 1988 in Germany (Karch & Bielaszewska, 2001), the earliest report of infection with this pathogen in the United Kingdom occurred in Scotland in 2002, when it was isolated from a five year old child with HUS (Allison, 2002). There were subsequent reports of single cases of infection with SF VTEC O157:NM in Scotland and England in 2003 and 2004, respectively (HPA, 2006; Locking *et al.*, 2004). However, between April and May 2006, eighteen cases of SF VTEC O157:NM were identified in Scotland, thirteen of which were associated with a nursery (Editorial Team, 2006). A further two cases of SF VTEC O157:NM were identified in August 2006.

There is some evidence to suggest that SF VTEC O157:NM strains are more frequently associated with HUS than NSF VTEC O157:H7 strains. For example, in Germany the probability of development of HUS after infection with SF VTEC O157:NM is 1:2, whereas after infection with NSF VTEC O157:H7 this ratio is 1:6 (M. Bielaszewska and H. Karch, personal communication). A similar situation appears to be the case in Scotland, where ten out of twenty cases of SF VTEC O157:NM infection identified between April and August 2006 progressed to HUS. Whilst host susceptibility may differ between outbreaks, it is also possible that SF VTEC O157:NM strains are more virulent than NSF VTEC O157:H7 strains.

The objective of this study was to compare both genotypic and phenotypic characteristics of SF VTEC O157:NM and NSF VTEC O157:H7 isolates recovered from cases of infection in Scotland to identify bacterial factors which may contribute to the increased association of SF VTEC O157:NM isolates with HUS.

Chapter 5

Genotypic and phenotypic characterisation of sorbitol-fermenting *Escherichia coli* O157:NM isolates

The observation that SF VTEC O157:NM strains are more frequently associated with HUS than NSF VTEC O157:H7 strains prompted an investigation of bacterial characteristics that may contribute to this. Since verocytotoxins are responsible for the serious consequences of VTEC O157 infection including HUS, it is possible that any increased pathogenicity of SF VTEC O157:NM strains could be accounted for by: (1) a more potent toxin variant; (2) increased toxin expression; (3) colonisation of the human intestine at higher levels leading to a greater exposure to toxin. This chapter describes the genotypic and phenotypic characterisation of SF VTEC O157:NM and NSF VTEC O157:H7 isolates, with a particular focus on whether SF VTEC O157:NM strains could be considered more virulent as a result of increased toxin expression or enhanced colonisation potential.

5.1 *E. coli* O157 isolates

Ten *E. coli* O157 isolates recovered from cases of human infection were collected for this study. A full description of these isolates is detailed in section 2.1.3 and Table 2.1(C). Briefly, six of these isolates were SF VTEC O157:NM recovered in Scotland; five were isolated during the 2006 Scottish outbreak cluster and one was isolated from a single case in 2003 (Locking *et al.*, 2004). The remaining four *E. coli* O157 isolates were NSF VTEC O157:H7, which were included for comparison. Three NSF VTEC O157:H7 isolates were recovered from separate Scottish outbreaks and the remaining isolate was the sequenced strain EDL933. Two representative SF VTEC O157:NM isolates (H2687 and H8824) and two NSF VTEC O157:H7 isolates (EDL933 and 1477/AI) were analysed in the phenotypic assays.

5.2 Confirmation of basic characteristics of *E. coli* O157 isolates

All isolates included in this research were confirmed to be *E. coli* serogroup O157 by immunostaining with O157 antisera. To confirm their phenotypic traits, isolates were plated on sorbitol MacConkey agar to test for sorbitol fermentation and inoculated into soft-agar (0.3% agar) to assess motility. The six SF VTEC O157:NM isolates fermented sorbitol within 24 h of incubation at 37°C on sorbitol MacConkey agar and were verified as non-motile. The NSF VTEC O157:H7 isolates did not ferment sorbitol and were confirmed to be motile. Although the SF VTEC

O157:NM isolates were non-motile, their gene encoding the flagellin subunit (*fliC*) was detected and characterised using a *fliC* PCR-restriction fragment length polymorphism (RFLP) method (Fields *et al.*, 1997). The six SF VTEC O157:NM isolates shared a *fliC*-RFLP pattern that was identical to the four NSF VTEC O157:H7 isolates (Figure 5.1). This demonstrated that the SF VTEC O157:NM isolates recovered from infections in Scotland possess the *fliC* gene encoding the H7 antigen.

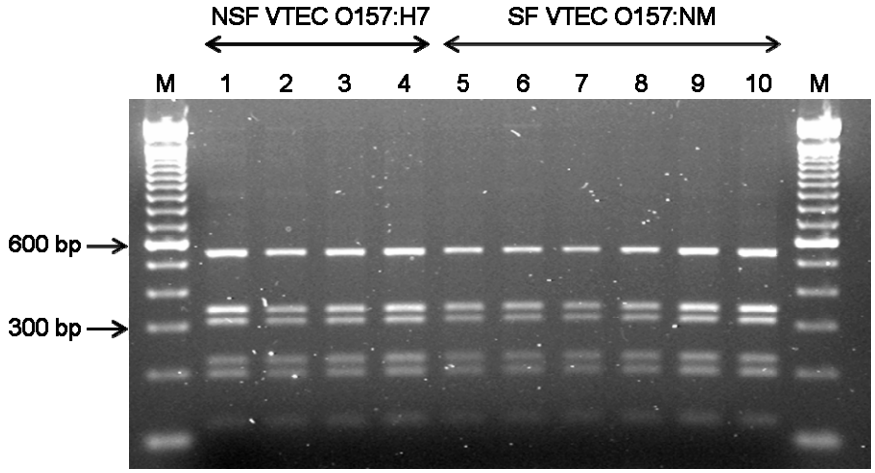


Figure 5.1. PCR-restriction fragment length polymorphism of the *fliC* gene from VTEC O157 isolates. Agarose gel electrophoresis of *fliC* PCR products after restriction with *Rsa*I from NSF VTEC O157:H7 isolates EDL933 (lane 1), H77 (lane 2), H511 (lane 3) and 1477/AI (lane 4) and SF VTEC O157:NM isolates H2687 (lane 5), H8824 (lane 6), H8432 (lane 7), H8489 (lane 8), H8757 (lane 9) and H8478 (lane 10). Lane M, 100 bp molecular size marker.

Multiplex PCR for the detection of genes encoding verocytotoxin 1 (vtx_1), verocytotoxin 2 (vtx_2), intimin (*eae*) and enterohaemolysin (*hlyA*) demonstrated that the SF VTEC O157:NM isolates were positive for vtx_2, *eae* and *hlyA* and negative for vtx_1. The three NSF VTEC O157:H7 isolates from Scottish outbreaks were also positive for vtx_2, *eae* and *hlyA* and negative for vtx_1. VTEC O157:H7 EDL933 was confirmed to be positive for vtx_1, vtx_2, *eae* and *hlyA*.

5.3 Nucleotide sequencing of *vtx*$_2$

To investigate whether the SF VTEC O157:NM isolates produce a different, possibly more potent, Vtx2 variant to NSF VTEC O157:H7, the nucleotide sequences of *vtx*$_2$ were determined for the six Scottish SF VTEC O157:NM isolates. The sequences from the five isolates obtained from the 2006 Scottish cluster (GenBank accession numbers are detailed in Table 2.3) were identical and the same as those published for three previously characterised SF VTEC O157:NM strains (Bielaszewska *et al.*, 2006). Their A and B subunit genes differed from the published *E. coli* EDL933 sequence by seven nucleotides and one nucleotide, respectively, and encode a Vtx2 protein which differed from Vtx2 from EDL933 by a single amino acid residue in each of the subunits. The *vtx*$_2$ sequence from SF VTEC O157:NM isolate H2687 (GenBank accession number EU526759) differed from EDL933 by a single nucleotide in the A subunit. The B subunits were identical. This SF VTEC O157:NM isolate had a predicted protein sequence for Vtx2 identical to that of EDL933.

5.4 Vero cell cytotoxicity assays

To determine whether SF VTEC O157:NM isolates produced greater quantities of Vtx2 than NSF VTEC O157:H7 isolates, the production of Vtx by the two VTEC O157 groups was investigated using the Vero cell cytotoxicity assay. A single SF VTEC O157:NM isolate from the 2006 Scottish outbreak cluster (H8824) was analysed along with SF VTEC O157:NM isolate H2687 recovered from a single case in 2003 and compared with NSF VTEC O157:H7 isolates EDL933 and 1477/AI. *E. coli* O157:H7 TUV93-0, a toxin-negative derivative of EDL933, was included as a negative control. The levels of Vtx production by each isolate was assessed following their culture in MEM without and after induction with mitomycin C and the results are shown in Figure 5.2.

One-way analysis of variance analysis of Vero cell survival following exposure to basal levels of toxin in cultures filtrates from the four VTEC O157 uninduced cultures demonstrated significant variation ($P < 0.001$). The source of this variation is almost entirely due to EDL933, which has a different verocytotoxin background

(Vtx1 and Vtx2) to the other three VTEC O157 isolates (Vtx2 alone). Induction with mitomycin C reduced Vero cell survival for all toxin-positive isolates tested, but there was no significant difference in Vero cell survival between the four VTEC O157 isolates examined following induction (P = 0.723). Therefore, under the *in vitro* conditions tested there was no evidence of increased toxin expression and activity from the SF VTEC O157:NM isolates in comparison with the NSF VTEC O157:H7 isolates.

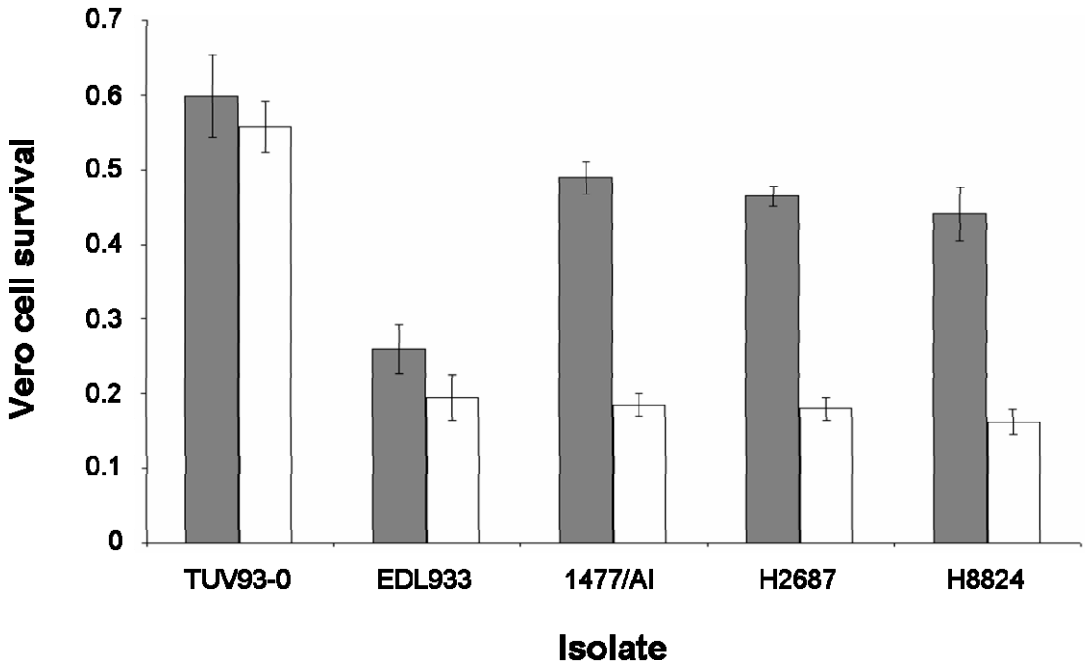


Figure 5.2. Vero cell cytotoxicity of culture filtrates from VTEC O157 isolates. NSF VTEC O157:H7 isolates (EDL933 and 1477/AI) and SF VTEC O157:NM isolates (H2687 and H8824) were cultured in MEM-HEPES with (unshaded bars) and without (shaded bars) mitomycin C (0.5 μg/ml). TUV93-0, a Vtx-negative derivative of EDL933, serves as a negative control. Viable Vero cells, following 72 h of exposure to culture filtrates, were quantified by crystal violet staining and subsequent absorbance measurement of crystal violet in solution. Vero cell survival is expressed relative to untreated controls not exposed to bacterial culture filtrates. Error bars indicate standard errors of the means.

5.5 LEE protein secretion

Type III secretion-based intimate attachment is likely to be important for intestinal colonisation and both SF VTEC O157:NM and NSF VTEC O157:H7 isolates possess a type III secretion system. To investigate if there are differences in the level of secretion of LEE-encoded proteins between the two VTEC O157 groups, the proteins present in the supernatants of three NSF VTEC O157:H7 and six SF VTEC O157:NM isolates cultured in MEM-HEPES were analysed. It is known that MEM-HEPES is permissive for LEE-protein secretion by *E. coli* O157:H7 (Roe *et al.*, 2003) and *E. coli* O157:H7 Walla-3, a Vtx-negative strain which secretes high levels of LEE-encoded proteins, was included as a positive control. The isolates were cultured in MEM-HEPES to an OD_{600} of 0.8 and supernatant proteins were TCA precipitated, separated by SDS-PAGE and visualised by Colloidal blue staining. The isolates were cultured on three separate occasions and representative secreted protein profiles are shown in Figure 5.3.

None of the VTEC O157 isolates secreted LEE-encoded proteins at levels which could be detected by Colloidal blue staining. Therefore, there was no evidence of differences in the level of LEE-encoded protein secretion from the SF VTEC O157:NM isolates in comparison with the NSF VTEC O157:H7 isolates.

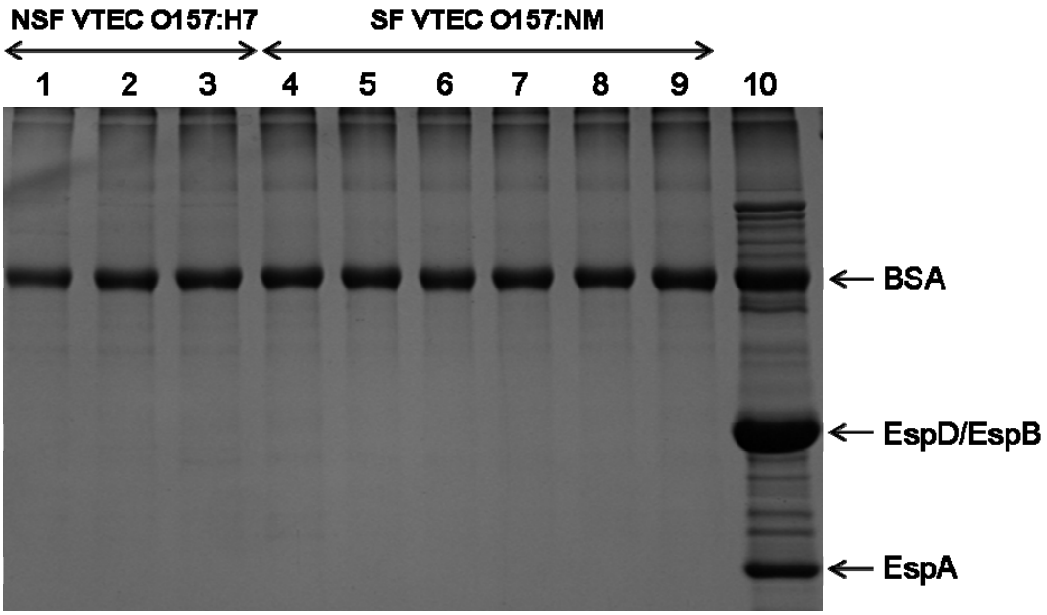


Figure 5.3. LEE-encoded protein secretion by VTEC O157 isolates. Secreted protein profiles from NSF VTEC O157:H7 isolates H77 (lane 1), H511 (lane 2) and 1477/AI (lane 3) and SF VTEC O157:NM isolates H2687 (lane 4), H8824 (lane 5), H8432 (lane 6), H8489 (lane 7), H8757 (lane 8) and H8478 (lane 9). *E. coli* O157:H7 Walla-3 was included as a high secretor positive control (lane 10). Bacteria were cultured in MEM-HEPES to an OD_{600} of 0.8 and supernatant proteins were TCA precipitated, separated by SDS-PAGE and visualised by staining with Colloidal blue. The positions of the co-precipitant BSA (66 kDa) and the secreted proteins EspD/EspB (~ 33 kDa) and EspA (~ 22 kDa) are indicated.

5.6 Adherence assays

In addition to type III secretion-based intimate attachment, adherence mediated by surface-expressed factors such as fimbriae may also be important for intestinal colonisation.

5.6.1 Adherence to Caco-2 cells

To investigate if there are differences in the capacities of the two VTEC O157 groups to adhere to human intestinal epithelial cells, NSF VTEC O157:H7 isolates EDL933 and 1477/AI and SF VTEC O157:NM isolates H2687 and H8824 were tested for their capacity to bind to Caco-2 cells within 2 h. Bacteria were cultured at 37ºC under two different conditions; namely, on CFA agar plates and in CDMT broth, which have both been used previously to promote fimbrial expression (Brunder *et al.*, 2001; Evans *et al.*, 1977). The adherence of the VTEC O157 isolates to Caco-2 cells is shown in Figure 5.4A. Significantly higher levels of adherence (fifty-fold) were observed for the SF VTEC O157:NM isolates compared with the NSF VTEC O157:H7 isolates when cultured on CFA agar, whereas little difference was observed between the two groups when cultured in CDMT broth (Figure 5.4A).

Microscopy revealed that the SF VTEC O157:NM isolates cultured on CFA agar at 37ºC adhered to Caco-2 cells in aggregates whereas the NSF VTEC O157:H7 isolates cultured in this way did not. Images showing the adherence of the VTEC O157 isolates, cultured on CFA agar, to Caco-2 cells are provided in Figure 5.4B.

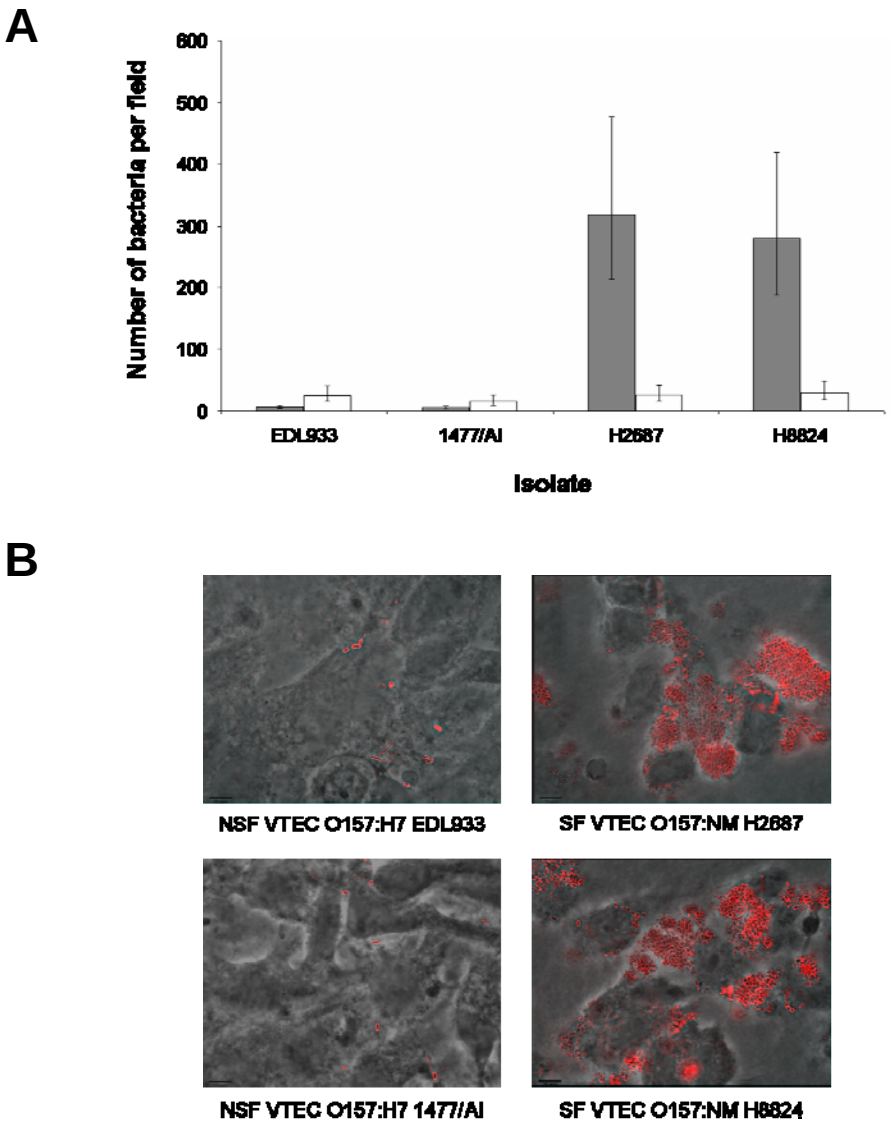


Figure 5.4. Adherence of VTEC O157 isolates to Caco-2 cells. (A) NSF VTEC O157:H7 isolates (EDL933 and 1477/AI) and SF VTEC O157:NM isolates (H2687 and H8824) were cultured on CFA agar (shaded bars) and in CDMT broth (unshaded bars) at 37°C and adherence assays were performed at 37°C for 2 h. Microscopy was used to determine the number of bacteria per field for at least 20 fields. Adherence assays for each isolate were performed on at least three separate occasions. Data were $\log_{10}$ transformed and analysed within mixed models using the REML directive. The bars represent back-transformed predicted means and error bars define 95% confidence interval values. (B) Visualisation of adherence to Caco-2 cells by immunofluorescence microscopy. The indicated isolates were cultured on CFA agar at 37°C and the micrographs show bacteria (stained red) adherent to Caco-2 cells after 2 h of incubation at 37°C. Slides were examined using an x100 objective and images were captured with Leica software.

5.6.2 Biofilm assays

The observed difference in the level of adherence to an intestinal epithelial cell line between SF VTEC O157:NM isolates and NSF VTEC O157:H7 isolates following culture on CFA agar and the fact that the SF VTEC O157:NM isolates, but not the NSF VTEC O157:H7 isolates, autoaggregated following CFA culture prompted an investigation of the ability of the two VTEC O157 groups to adhere to an abiotic surface. Isolates were cultured in polystyrene microtitre plates in LB, CFA and CDMT broths and adherence assays were performed at 28ºC or 37ºC for 24 h or 48 h. The extent of bacterial adherence to microtitre well surfaces was determined by crystal violet staining and the results are shown in Figure 5.5.

The SF VTEC O157:NM isolates demonstrated increased adherence to polystyrene microtitre plates in comparison with the NSF VTEC O157:H7 isolates under the different conditions tested (Figure 5.5A and B). It was evident that culturing the two SF VTEC O157:NM isolates in CDMT broth promoted biofilm formation but did not increase adherence to Caco-2 cells. This indicates that the same combination of factors is not required for the two different adherence phenotypes.

5.7 Analysis of fimbriae expression

The observation that the SF VTEC O157:NM isolates adhered to Caco-2 cells at significantly higher levels than NSF VTEC O157:H7 isolates following culture on CFA agar prompted an investigation of the factor(s) responsible for this. At least sixteen putative fimbrial operons have been identified in NSF VTEC O157:H7 (Hayashi *et al.*, 2001; Low *et al.*, 2006; Perna *et al.*, 2001), including the well-characterised adhesins type 1 fimbriae and curli. While the capacity to express many of these fimbrial adhesins appears to have been lost by NSF VTEC O157:H7 strains (Low *et al.*, 2006), SF VTEC O157:NM strains may be different. In addition, SF VTEC O157:NM uniquely possess the *sfp* gene cluster which encodes novel Sfp fimbriae (Brunder *et al.*, 2001).

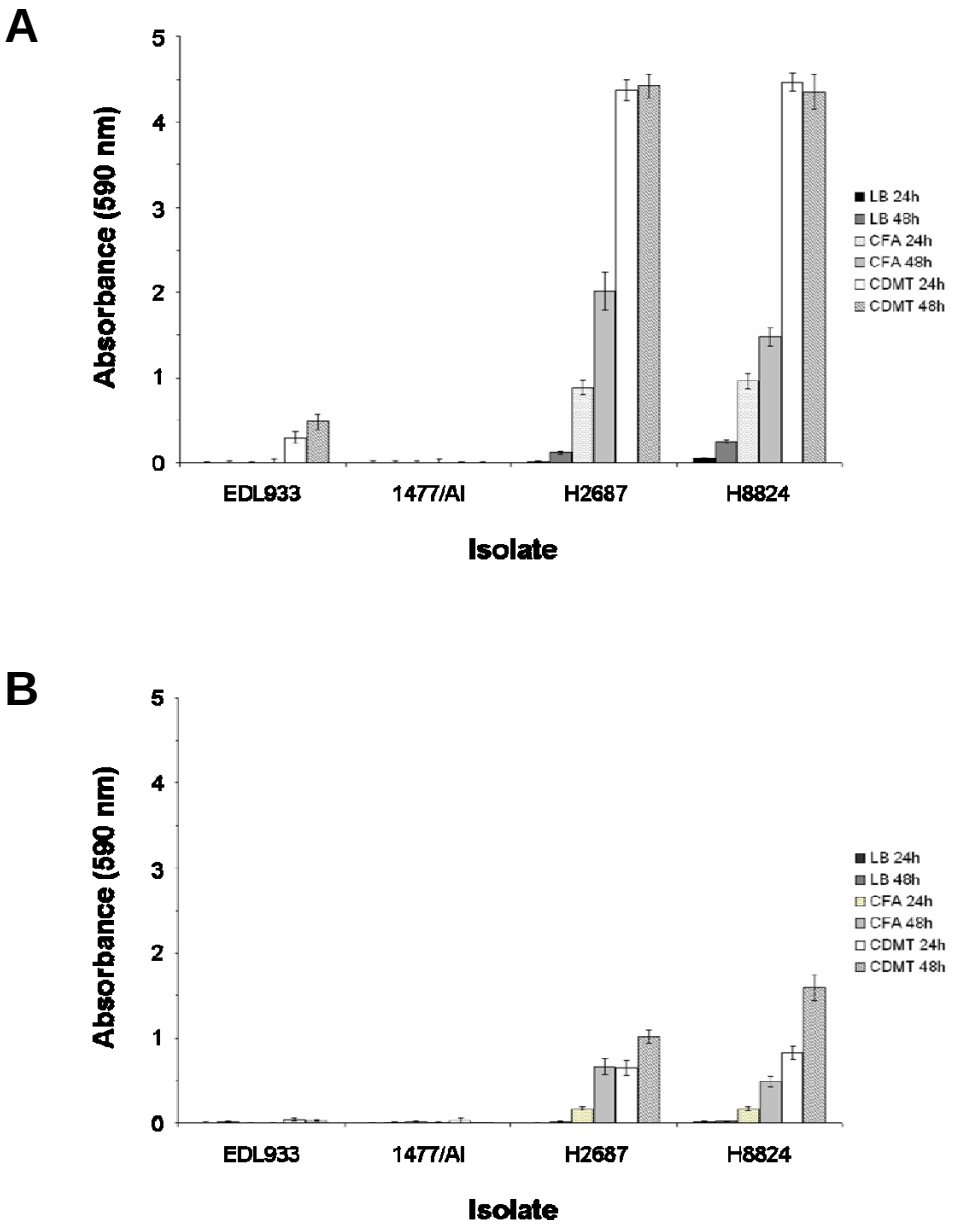


Figure 5.5. Adherence of VTEC O157 isolates to polystyrene microtitre plates. NSF VTEC O157:H7 isolates (EDL933 and 1477/AI) and SF VTEC O157:NM isolates (H2687 and H8824) were cultured in LB, CFA and CDMT broths and adherence assays were performed for 24 h and 48 h at (A) 28°C and (B) 37°C. Bacterial adherence was quantified by crystal violet staining of bacteria and subsequent absorbance measurement (590 nm) of released crystal violet. Background levels were determined for sterile media and were subtracted from the other values. Assays were performed on three separate occasions. The bars represent the average absorbance of released crystal violet for each isolate and error bars indicate standard errors of the means.

5.7.1 Analysis of type 1 fimbriae expression by NSF VTEC O157:H7 and SF VTEC O157:NM

NSF *E. coli* O157:H7 strains have been shown to contain a 16 bp deletion in the *fim* switch that controls type 1 fimbriae expression (Li *et al.*, 1997; Roe *et al.*, 2001), thus preventing inversion of the *fim* switch to the on orientation and the expression of type 1 fimbriae. A PCR designed to detect the presence of this deletion (Li *et al.*, 1997) was used to analyse the two groups of VTEC O157 strains and the results are shown in Figure 5.6A. The SF VTEC O157:NM isolates did not contain the deletion (Figure 5.6A, lanes 5 to 10), whereas the deletion was present in the NSF VTEC O157:H7 isolates tested (Figure 5.6A, lanes 1 to 4).

The status of type 1 fimbriae expression was then examined using the *fim* switch orientation assay and yeast cell agglutination. The two groups of VTEC O157 isolates were initially cultured under conditions optimal for expression of type 1 fimbriae (3 day subculture in LB broth, statically at 37°C). PCR and restriction digestion analysis of the *fim* switch found that the SF VTEC O157:NM isolates had the *fim* switch in both the on and off orientations whereas the NSF VTEC O157:H7 isolates only had the *fim* switch in the off orientation (Figure 5.6B). The presence of functional type 1 fimbriae was assessed by yeast cell agglutination and only the SF VTEC O157:NM isolates exhibited agglutination of yeast cells (Figure 5.6B), which was found to be mannose-sensitive (data not shown). Taken together, these results demonstrate that, in contrast to NSF VTEC O157:H7, SF VTEC O157:NM strains can express functional type 1 fimbriae. However, when SF VTEC O157:NM isolates were cultured on CFA agar at 37°C, as for the adherence assays, the majority of the bacterial population had the *fim* switch in the off orientation (Figure 5.6C, lanes 3 and 4).

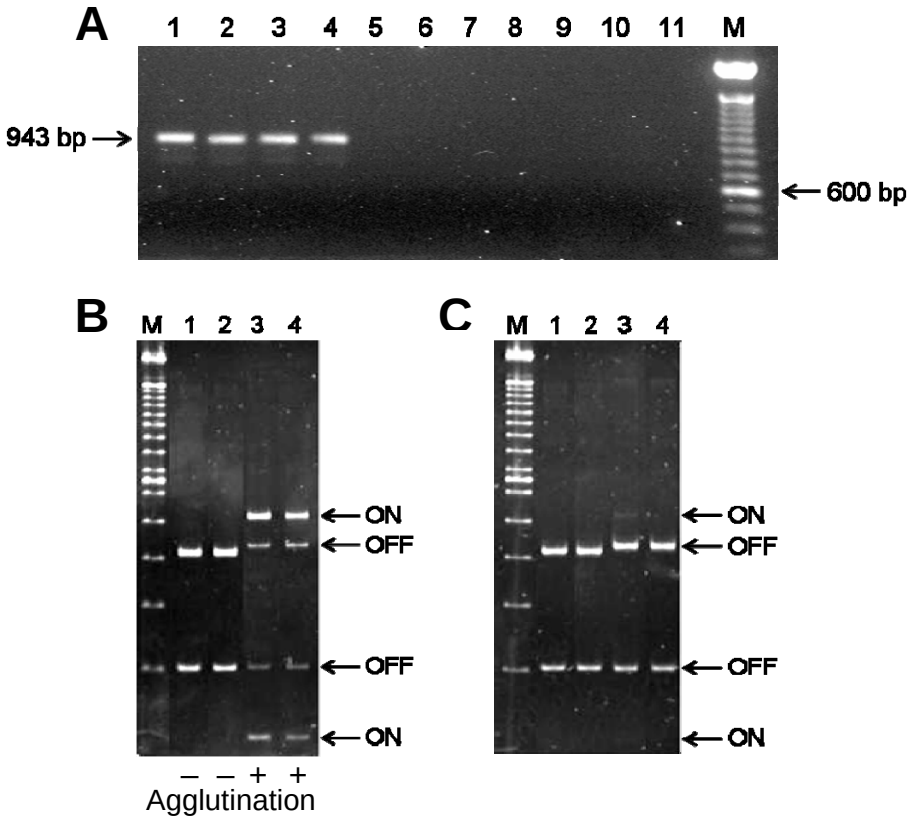


Figure 5.6. Analysis of type 1 fimbriae expression by VTEC O157 isolates. (A) PCR analysis of the *fim* switch deletion using VTEC O157:H7-specific primers. NSF VTEC O157:H7 isolates EDL933 (lane 1), H77 (lane 2), H511 (lane 3) and 1477/AI (lane 4) generate a 943 bp product indicating the presence of the deletion that is absent in the SF VTEC O157:NM isolates H2687 (lane 5), H8824 (lane 6), H8432 (lane 7), H8489 (lane 8), H8757 (lane 9) and H8478 (lane 10). Lane 11, DNA negative (PCR control); lane M, 100 bp molecular size marker. (B and C) PCR and restriction digestion of the *fim* switch from isolates indicating the expression status within the bacterial population. (B) The isolates were cultured under conditions optimal for type 1 fimbriae expression (3 day subculture in LB broth, statically at 37°C). The results of the yeast cell agglutination assay are summarised below the gel image. The *fim* switch was detected in both the on and off orientations for the two SF VTEC O157:NM isolates examined [H2687 (lane 3) and H8824 (lane 4)] but only in the off orientation for the two NSF VTEC O157:H7 isolates analysed [EDL933 (lane 1) and 1477/AI (lane 2)]. (C) The isolates were cultured on CFA agar plates. The *fim* switch in the on orientation was only detected in one SF VTEC O157:NM isolate (H2687, lane 3) but not in the other (H8824, lane 4) nor in the two NSF VTEC O157:H7 isolates [EDL933 (lane 1) and 1477/AI (lane 2)]. Lane M, 100 bp molecular size marker.

5.7.1.1 Type 1 fimbriae inhibition adherence assays

To evaluate the contribution of type 1 fimbriae in the adherence of SF VTEC O157:NM isolates to Caco-2 cells following culture on CFA agar, inhibition assays were carried out in the presence of 3% (w/v) α-$_D$-mannose. The addition of mannose reduced adherence of the two SF VTEC O157:NM isolates tested (Figure 5.7) but this reduction was not statistically significant.

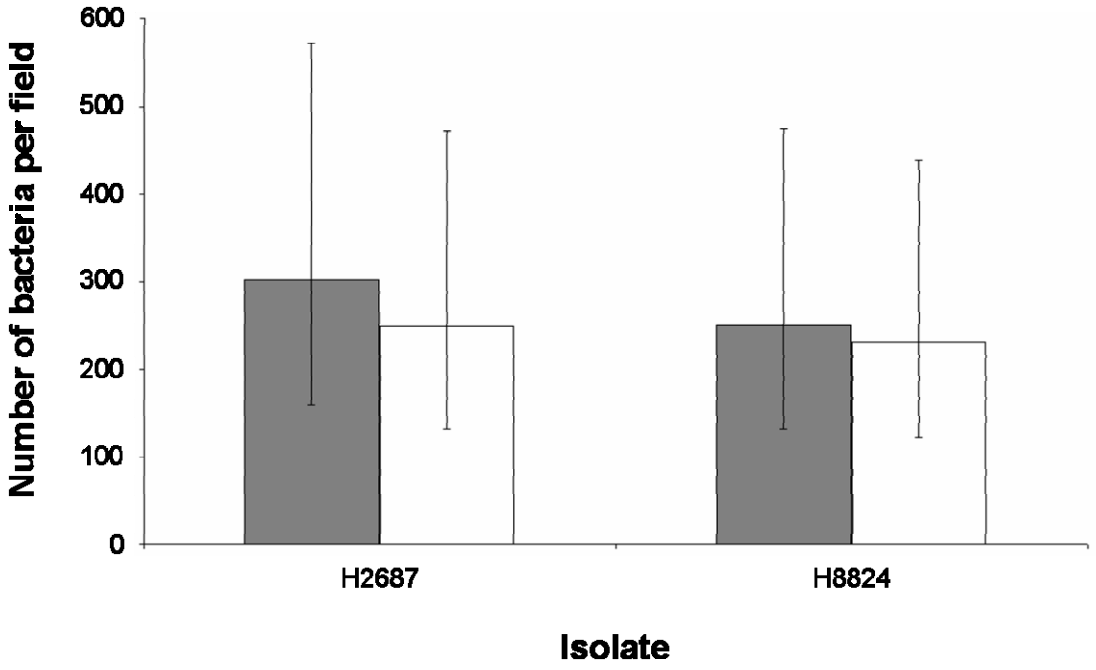


Figure 5.7. Contribution of type 1 fimbriae to Caco-2 cell adherence. SF VTEC O157:NM isolates H2687 and H8824 were cultured on CFA agar at 37°C and Caco-2 adherence assays were performed in the absence (shaded bars) and presence (unshaded bars) of 3% (w/v) α-$_D$-mannose, which will inhibit type 1 fimbrial adherence. Microscopy (using an x100 objective) was used to determine the number of bacteria per field for at least 20 fields. Adherence assays for each isolate were performed on three separate occasions. Data were $\log_{10}$ transformed and analysed within mixed models using the REML directive. The bars represent back-transformed predicted means and error bars define 95% confidence interval values.

5.7.2 Analysis of Sfp fimbriae expression by NSF VTEC O157:H7 and SF VTEC O157:NM

SF VTEC O157:NM strains possess a unique fimbrial gene cluster on their large plasmid, pSFO157, which encodes novel Sfp fimbriae (Brunder *et al.*, 2001). The expression of Sfp fimbriae on the surface of bacteria cultured on CFA agar and in CDMT broth at 37°C was investigated by immunostaining with serum raised to the main subunit of these fimbriae, SfpA (Brunder *et al.*, 2001), kindly provided by Prof H. Karch. When the isolates were cultured on CFA agar at 37°C, a proportion of the SF VTEC O157:NM bacteria exhibited fluorescent surface staining whereas there was no antibody recognition of NSF VTEC O157:H7 bacteria (Figure 5.8A). There was no surface staining of either VTEC O157 group when the bacteria were cultured in CDMT broth (data not shown).

Further investigation of Sfp fimbriae expression was achieved by analysing *sfpA* promoter activity. The promoter region for *sfpA* from SF VTEC O157:NM isolate H8824 was fused to *gfp*, creating a translational fusion. The fusion was transformed into the appropriate VTEC O157 background and transformants were cultured on CFA agar and in CDMT broth, both containing chloramphenicol (CAM), at 37°C. Total GFP produced by the population was determined and the results are shown in Figure 5.8B. There were no significant differences in *sfpA* promoter activity between the NSF VTEC O157:H7 isolate 1477/AI and the SF VTEC O157:NM isolate H8824 when they were cultured on CFA agar or in CDMT broth. However, *sfpA* promoter activity in SF VTEC O157:NM isolate H8824 was found to be significantly higher ($P = 0.0015$) following culture on CFA agar compared to in CDMT broth (Figure 5.8B). Taken together, these results indicate that the SF VTEC O157:NM isolate tested expresses Sfp fimbriae at 37°C on CFA agar.

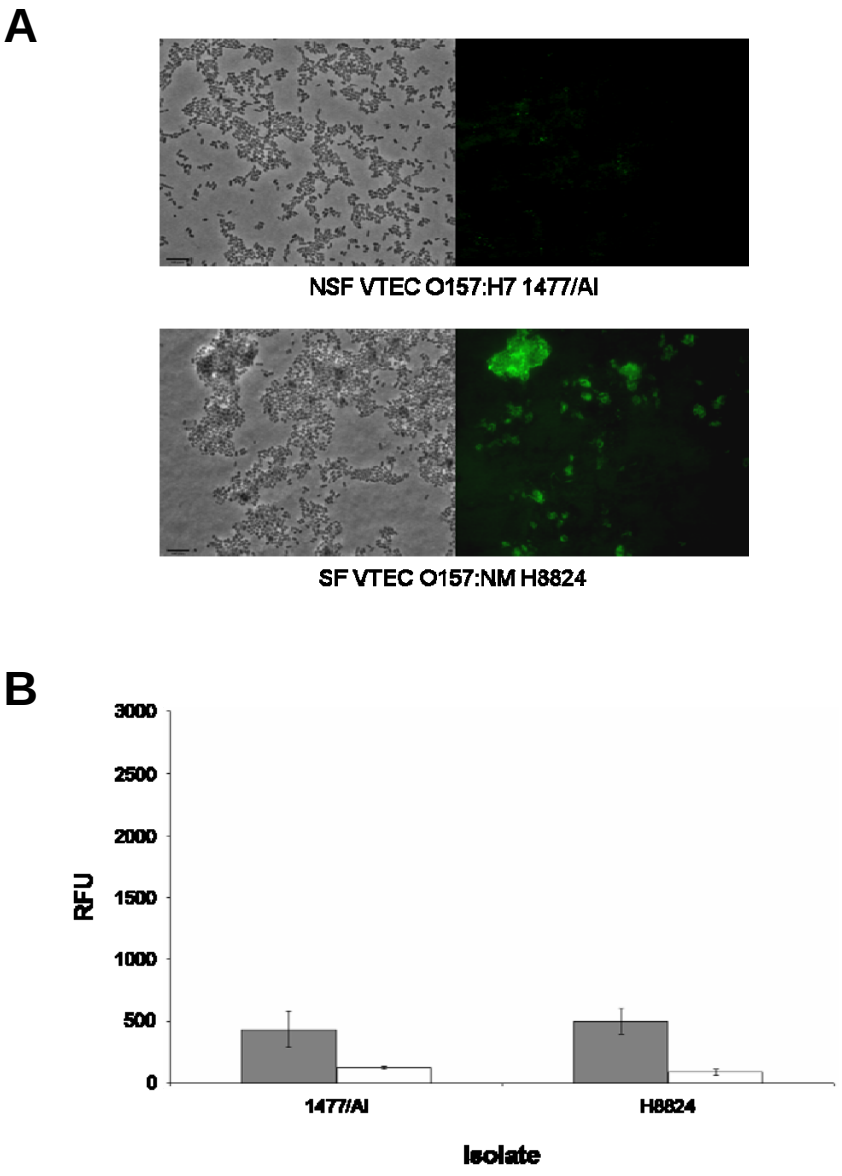


Figure 5.8. Analysis of Sfp fimbriae expression by VTEC O157 isolates. (A) Detection of SfpA by immunofluorescence microscopy. Phase-contrast (left panel) and fluorescence (right panel) micrographs are shown for the indicated VTEC O157 isolates stained for SfpA, using anti-SfpA serum, following culture on CFA agar at 37°C. (B) *sfpA* promoter activity in VTEC O157 isolates. The indicated isolates were transformed with a plasmid-based promoter-GFP fusion and expression from the *sfpA* promoter was determined following the culture of transformed isolates on CFA-CAM agar (shaded bars) and in CDMT-CAM broth (unshaded bars) at 37°C. Background fluorescence levels were determined for the promoter-less plasmid pAJR70 and subtracted. The data are expressed as relative fluorescence units (RFU). Error bars indicate standard errors of the means.

5.7.2.1 Sfp fimbriae inhibition adherence assays

To evaluate the contribution of Sfp fimbriae in the adherence of SF VTEC O157:NM isolates to Caco-2 cells following culture on CFA agar at 37°C, inhibition assays were attempted using anti-SfpA serum at a 1:400 dilution. There was a slight reduction in adherence to Caco-2 cells by the two SF VTEC O157:NM isolates tested in the presence of anti-SfpA serum (Figure 5.9), but this was not statistically significant.

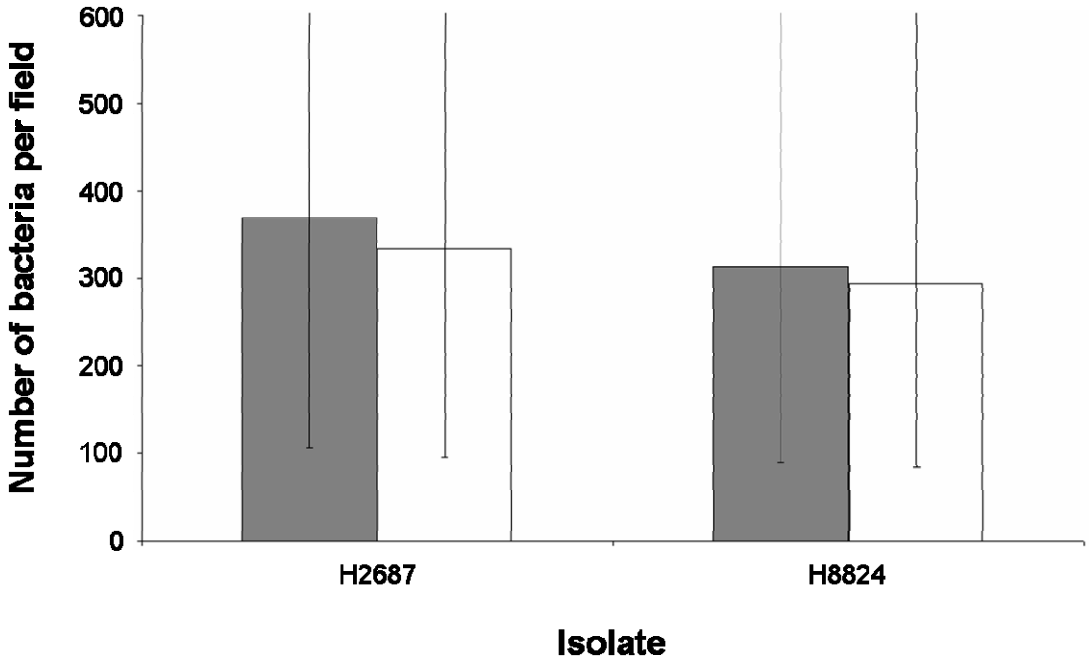


Figure 5.9. Contribution of Sfp fimbriae to Caco-2 cell adherence. SF VTEC O157:NM isolates H2687 and H8824 were cultured on CFA agar at 37°C and incubated in the absence (shaded bars) and presence (unshaded bars) of anti-SfpA serum. Microscopy was used to determine the number of bacteria per field for at least 20 fields. Adherence assays for each isolate were performed on three separate occasions. Data were $\log_{10}$ transformed and analysed within mixed models using the REML directive. The bars represent back-transformed predicted means and error bars define 95% confidence interval values.

5.7.3 Analysis of curli expression by NSF VTEC O157:H7 and SF VTEC O157:NM

The observation that the SF VTEC O157:NM isolates, but not the NSF VTEC O157:H7 isolates, autoaggregated following CFA culture prompted an investigation of curli expression by the two VTEC O157 groups. Curli are thin aggregative fimbriae and are an established adhesin (Barnhart & Chapman, 2006). However, while curli are expressed by many strains of *E. coli* (Barnhart & Chapman, 2006), the majority of NSF VTEC O157:H7 strains are considered not to express this adhesin under *in vitro* conditions (Cookson *et al.*, 2002; Uhlich *et al.*, 2001).

Congo red (CR) binding is a well established method to demonstrate surface curli expression by bacteria (Barnhart & Chapman, 2006; Hammar *et al.*, 1995; Uhlich *et al.*, 2001). The VTEC O157 isolates were cultured on CFA-CR indicator plates at 37°C and while the NSF VTEC O157:H7 isolates EDL933 and 1477/AI were negative, the SF VTEC O157:NM isolates H2687 and H8824 were positive (data not shown). Additional evidence for curli expression was provided by immunostaining with an anti-SEF17 monoclonal antibody which recognises curli, kindly provided by Dr R. La Ragione. For ease, this anti-SEF17 antibody will be referred to as "anti-curli" throughout the remainder of this thesis. When the isolates were cultured on CFA agar or in CFA broth at 37°C, the majority of aggregated SF VTEC O157:NM bacteria exhibited fluorescent surface staining, while the NSF VTEC O157:H7 bacteria were negative (Figure 5.10A). When the bacteria were cultured in CDMT broth, there was no surface staining of either VTEC O157 group (data not shown). Furthermore, analysis of curli main subunit (*csgBAC*) promoter activity, using a promoter-GFP translational fusion, revealed that the SF VTEC O157:NM isolates had significantly higher ($P < 0.001$) levels of expression from the *csgBAC* curli promoter compared with the NSF VTEC O157:H7 isolates when cultured on CFA agar at 37°C (Figure 5.10B). Taken together, these data indicate that the SF VTEC O157:NM isolates tested express curli at 37°C on CFA agar.

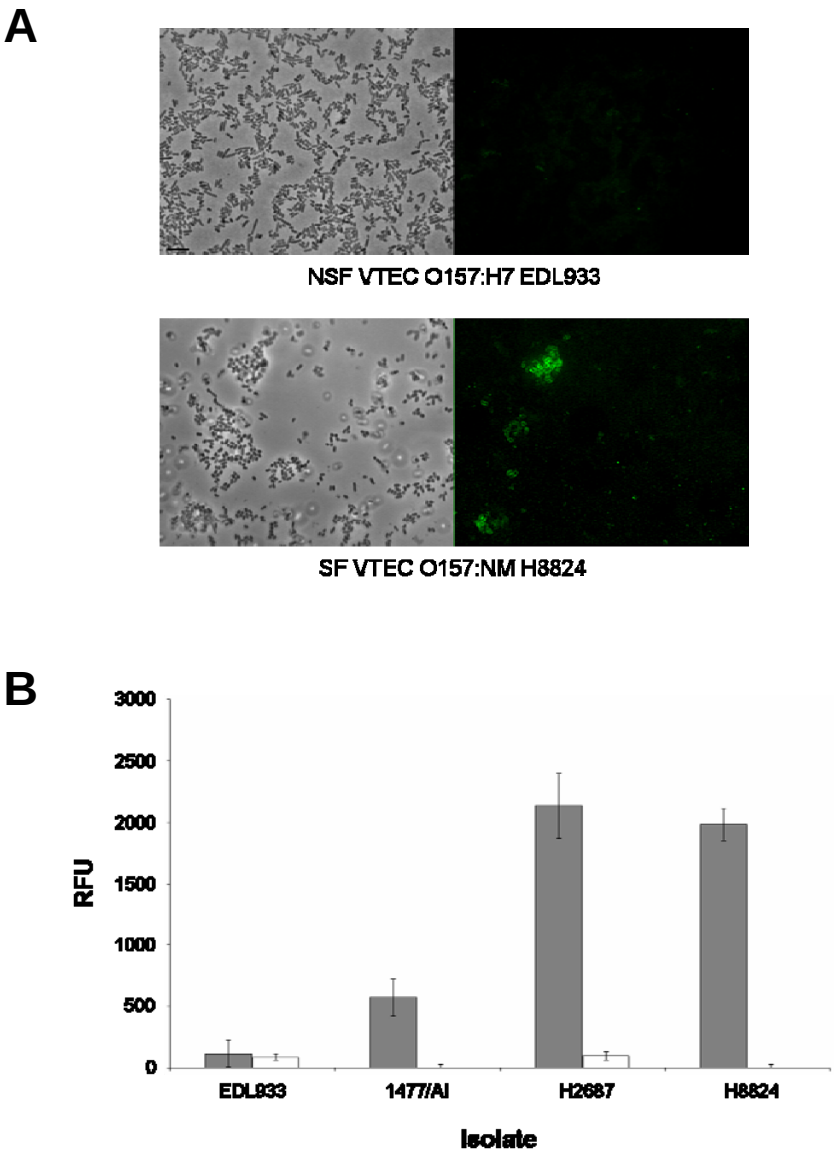


Figure 5.10. Analysis of curli expression by VTEC O157 isolates. (A) Detection of curli by immunofluorescence microscopy. Phase-contrast (left panel) and fluorescence (right panel) micrographs are shown for the indicated VTEC O157 isolates stained for curli using an anti-curli monoclonal antibody following culture on CFA medium at 37°C. (B) *csgBAC* curli promoter activity in VTEC O157 isolates. NSF VTEC O157:H7 isolates (EDL933 and 1477/AI) and SF VTEC O157:NM isolates (H2687 and H8824) were transformed with a plasmid-based promoter-GFP fusion and expression from the *csgBAC* promoter was determined following the culture of transformed isolates on CFA-CAM agar (shaded bars) and in CDMT-CAM broth (unshaded bars). Background fluorescence levels were determined for the promoter-less plasmid pAJR70 and subtracted. The data are expressed as relative fluorescence units (RFU). Error bars indicate standard errors of the means.

5.7.3.1 The contribution of curli to SF VTEC O157:NM adherence

To evaluate the contribution of curli in the adherence of SF VTEC O157:NM isolates to Caco-2 cells, inhibition assays were carried out using an anti-curli monoclonal antibody at a 1:100 dilution. The SF VTEC O157:NM isolates cultured on CFA agar at 37°C demonstrated a significant inhibition of adherence to Caco-2 cells in the presence of the antibody (Figure 5.11A). This indicated that, under these conditions, curli were an important factor in the increased adherence of SF VTEC O157:NM isolates to Caco-2 cells. To further substantiate the role of curli in the adherence, adherence assays comparing a curli deletion mutant with its wild-type parent were performed. The curli mutant was created by Ms T. Dransfield (University of Edinburgh) by deleting *csgBA* from SF VTEC O157:NM isolate H8824 by allelic exchange. The *csgBA* deletion strain did not bind CR and was negative for curli expression as determined by immunostaining (data not shown). This deletion strain could be complemented for CR binding and curli expression using a plasmid created by Ms T. Dransfield, in which the *csgBAC* genes were cloned into pWSK29, allowing *csgBAC* induction. The adherence of the *csgBA* deletion strain to Caco-2 cells was significantly reduced ($P < 0.001$) compared to the parent strain and was equivalent to the non-curli-expressing NSF VTEC O157:H7 EDL933 (Figure 5.11B).

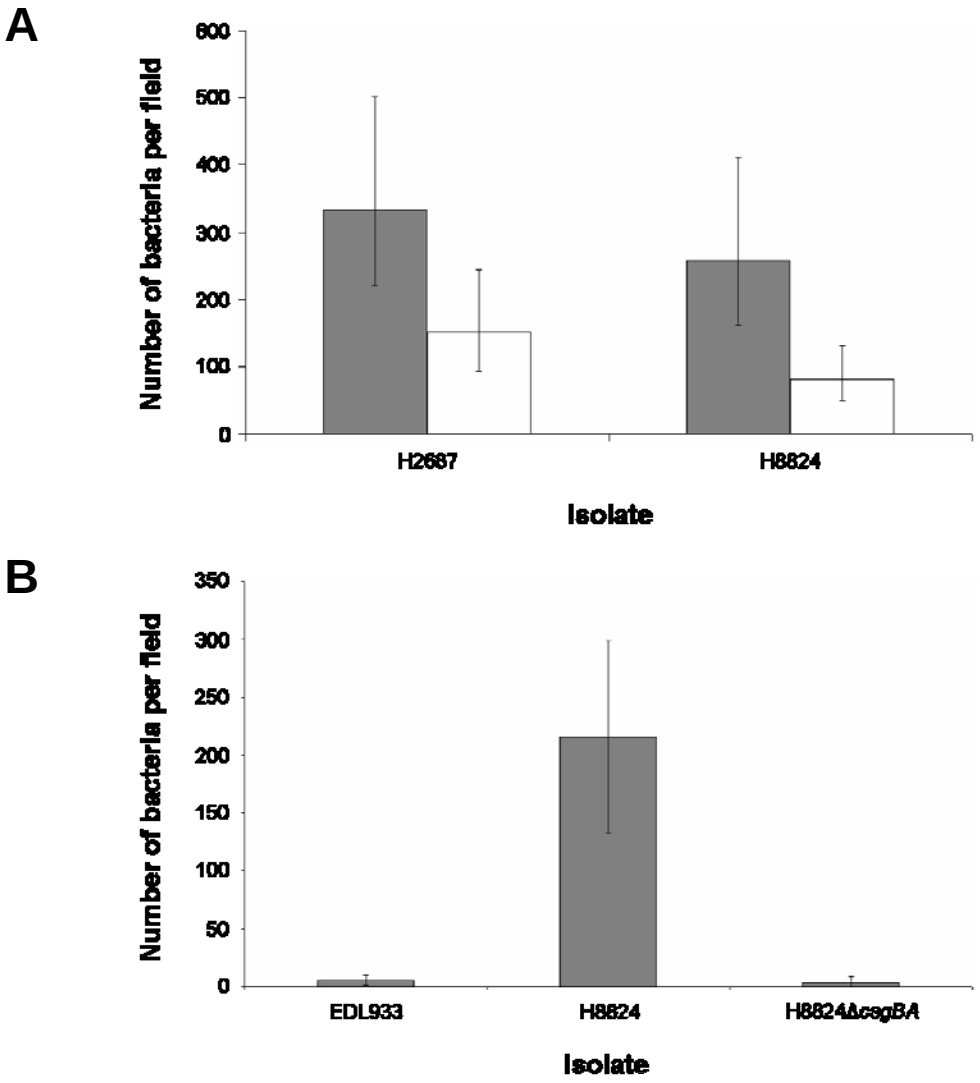


Figure 5.11. Contribution of curli to Caco-2 cell adherence. (A) Inhibition of adherence using anti-curli antibody. SF VTEC O157:NM isolates H2687 and H8824 were cultured on CFA agar at 37°C and incubated in the absence (shaded bars) and presence (unshaded bars) of anti-curli monoclonal antibody. Microscopy was used to determine the number of bacteria per field for 30 fields. Data were $\log_{10}$ transformed and analysed within mixed models using the REML directive. The bars represent back-transformed predicted means and error bars define 95% confidence interval values. (B) Comparative adherence studies of a curli deletion mutant and wild-type isolates. SF VTEC O157:NM isolate H8824 was deleted for *csgBA* and was demonstrated not to bind CR or express curli. The adherence of this strain to Caco-2 cells was compared with its parent and NSF VTEC O157:H7 EDL933. Microscopy was used to determine the number of bacteria per field for 30 fields. The bars represent the average number of bacteria per field and error bars define standard deviations.

5.8 Discussion

SF VTEC O157:NM strains have emerged as significant human pathogens in continental Europe, causing diarrheal disease often resulting in life-threatening HUS. Of particular concern is that 10 out of 20 Scottish patients infected with SF VTEC O157:NM in 2006 progressed to HUS. This is consistent with findings in Germany, where humans infected by these strains are more likely to progress to HUS. This compares to NSF VTEC O157:H7 infections that are typically associated with HUS at lower frequencies (5 to 10%) (Griffin & Tauxe, 1991; Mead & Griffin, 1998). As clinical cases with SF VTEC O157:NM are uncommon, host factors in these cases may be responsible for a bias toward HUS. These include age, clinical history or treatment issues such as the administration of antibiotics and/or anti-motility agents. However, the aim of this research was to investigate the hypothesis that bacterial factors are the cause of the increased association of SF VTEC O157:NM with HUS. This chapter described the genotypic and phenotypic characterisation of NSF VTEC O157:H7 and SF VTEC O157:NM isolates to identify bacterial traits which may be responsible for the increased virulence of SF VTEC O157:NM strains.

Basic characterisation of six Scottish SF VTEC O157:NM isolates confirmed that they were nonmotile in soft agar but carried the *fliC* gene encoding the H7 antigen. This is in agreement with previous characterisation of the *fliC* gene in European SF VTEC O157:NM strains (Bielaszewska *et al.*, 2000; Monday *et al.*, 2004). Analysis of SF *E. coli* O157:NM strains recovered in Germany found that a 12 bp in-frame deletion in the *flhC* gene of the *flhDC* master regulator operon, which controls flagellum biosynthesis, was responsible for their nonmotility (Monday *et al.*, 2004). The Scottish SF VTEC O157:NM isolates examined in this study possessed the genes encoding verocytotoxin 2, intimin and enterohaemolysin. This is in accord with all previous studies on SF VTEC O157:NM strains (Bettelheim *et al.*, 2002; Bielaszewska *et al.*, 1998; Bielaszewska *et al.*, 2000; Eklund *et al.*, 2006; Gunzer *et al.*, 1992; Keskimaki *et al.*, 1998). The NSF VTEC O157:H7 isolates recovered from Scottish outbreaks, which were included in this research for comparison, were deliberately chosen because they also possessed these genes.

Previous research has shown that SF VTEC O157:NM strains possess a specific combination of virulence determinants, including vtx_2 as the sole *vtx* gene, *eae* encoding γ-intimin, and a large plasmid that carries the *hlyA*, *etp* and *sfp* genes but not *espP* and *katP* (Karch & Bielaszewska, 2001). There are notable differences in the virulence characteristics of SF VTEC O157:NM and NSF VTEC O157:H7 strains. While SF VTEC O157:NM strains harbour vtx_2 as the sole *vtx* gene, NSF VTEC O157:H7 strains can possess vtx_1, vtx_2 and vtx_{2c} genes, either alone or in combination (Karch & Bielaszewska, 2001). In contrast to NSF VTEC O157:H7, SF VTEC O157:NM strains contain a complete EHEC factor for adherence (*efa1*) gene, which is only rudimentarily present in NSF VTEC O157:H7 (Janka *et al.*, 2002) and the majority of SF VTEC O157:NM strains possess the cytolethal distending toxin-V (*cdt-V*) gene cluster, which is absent in most NSF VTEC O157:H7 strains (Janka *et al.*, 2003). Differences also exist between the large plasmids harboured by these two pathogens. The large plasmid of SF VTEC O157:NM strains lacks *espP* and *katP*, which encode a serine protease and a catalase-peroxidase, respectively, and carries the *sfp* fimbriae gene cluster instead (Brunder *et al.*, 2006). In addition, although both large plasmids contain the gene for enterohaemolysin (*hlyA*), there are differences in its expression. The *hlyA* gene is regularly expressed in NSF VTEC O157:H7 strains whereas the majority of SF VTEC O157:NM strains do not express this gene and are thus nonhaemolytic on enterohaemolysin agar (Ammon *et al.*, 1999; Bielaszewska *et al.*, 1998; Bielaszewska *et al.*, 2000; Eklund *et al.*, 2006; Keskimaki *et al.*, 1998; Orth *et al.*, 2006).

It is possible that a higher HUS rate following SF VTEC O157:NM infection compared to NSF VTEC O157:H7 infection could be accounted for by a more active toxin or the release of more toxin during infection. In the present study, the predicted protein sequence for Vtx2 produced by the SF VTEC O157:NM isolates was either identical to or differed by a single amino acid in the A and B subunits from Vtx2 from NSF VTEC O157:H7 EDL933. The levels of toxin released by isolates under induced and uninduced *in vitro* conditions were examined, and there was no evidence of increased toxin expression from the SF VTEC O157:NM isolates. These results support the findings of others as published work has indicated

that there are no significant differences in the Vtx2 proteins encoded by SF VTEC O157:NM and NSF VTEC O157:H7 strains (Bielaszewska *et al.*, 2006), and a report suggests that their toxicity levels may be similar (Gunzer *et al.*, 1992). Thus, toxin sequence variation and increased toxin release are unlikely to account for any differences in virulence between SF VTEC O157:NM and NSF VTEC O157:H7 strains. However, it is appreciated that *in vivo* conditions may result in expression differences not observed *in vitro*.

Alternatively, SF VTEC O157:NM strains may colonise the human intestine in higher numbers, resulting in increased exposure to toxin. To test this, the potential of SF VTEC O157:NM and NSF VTEC O157:H7 strains to adhere to a human colon carcinoma cell line were compared. Culture of the isolates on CFA agar plates at 37°C resulted in up to 50 times more SF VTEC O157:NM bacteria than NSF VTEC O157:H7 bacteria adhering to Caco-2 cells within 2 hours. While type III secretion-based intimate attachment is likely to be important for intestinal colonisation (Donnenberg *et al.*, 1993; Tacket *et al.*, 2000), type III secretion profiles and levels were similar for the isolates analysed in this study. Consequently, initial adherence is more likely to involve surface-expressed factors such as fimbriae and so the expression and importance of established adhesins was investigated.

Type 1 fimbriae are the most common fimbrial adhesin produced by *E. coli* (Leathart & Gally, 1998). However, NSF VTEC O157:H7 are unable to express type 1 fimbriae due to a 16 bp deletion in the *fim* switch that controls type 1 fimbriae expression (Li *et al.*, 1997; Roe *et al.*, 2001). This study demonstrated that SF VTEC O157:NM strains do not contain the 16 bp deletion in the *fim* switch and, when cultured under conditions optimal for the expression of type 1 fimbriae, they exhibited mannose-sensitive agglutination of yeast cells and the bacterial population had the *fim* switch in both the on and off orientations. Thus, in contrast to NSF VTEC O157:H7, SF VTEC O157:NM are able to express functional type 1 fimbriae. These results corroborate the recent findings of Shaikh *et al.* (2007). However, when SF VTEC O157:NM were cultured on CFA agar, as for the adherence assays, the majority of the bacterial population had the *fim* switch in the off orientation,

indicating that type 1 fimbriae were unlikely to be the main factor responsible for the increased adherence to Caco-2 cells under these conditions. This was substantiated in inhibition adherence assays performed in the presence of mannose.

SF *E. coli* O157:NM strains have been shown to possess a unique fimbrial cluster encoded on their large plasmid which mediates the expression of Sfp fimbriae (Brunder *et al.*, 2001). In this study, immunostaining with serum raised to SfpA showed surface staining of a proportion of SF VTEC O157:NM bacteria cultured on CFA agar at 37°C and promoter fusion analysis agreed with this result. However, inhibition adherence assays performed in the presence of anti-SfpA serum did not significantly reduce the adherence of SF VTEC O157:NM isolates to Caco-2 cells, implying Sfp fimbriae were not the main factor responsible for the increased adherence of SF VTEC O157:NM isolates following culture on CFA agar. It should be noted that inhibition assays were only performed using anti-SfpA serum at a 1:400 dilution because there was not enough serum available to test inhibition at higher concentrations. Recently published work demonstrates that Sfp fimbriae are expressed by SF VTEC O157:NM strains cultured under anaerobic conditions on solid media simulating the colonic environment and that the induction of Sfp fimbriae on wild-type SF VTEC O157:NM strains correlated with increased adherence to Caco-2 and HCT-8 cells (Musken *et al.*, 2008).

Many strains of *E. coli* express thin aggregative fimbriae called curli (Barnhart & Chapman, 2006). In this study, an initial investigation of curli expression by the SF VTEC O157:NM isolates demonstrated that they could bind CR when cultured on CFA agar at 37°C. This assay is commonly applied at 28°C to detect surface curli expression (Barnhart & Chapman, 2006; Uhlich *et al.*, 2001). The expression of curli by the SF VTEC O157:NM isolates cultured on CFA medium at 37°C was substantiated by immunostaining with an anti-curli monoclonal antibody and *csgBAC* promoter fusion analysis. In contrast, the majority of NSF *E. coli* O157:H7 strains are considered not to express curli under *in vitro* conditions (Cookson *et al.*, 2002; Uhlich *et al.*, 2001) and the results of this study support this. Curli are an established adhesin, and so their contribution to the increased adherence of SF VTEC O157:NM

isolates compared to NSF VTEC O157:H7 isolates was examined. Inhibition adherence assays demonstrated that there was a significant inhibition of adherence of SF VTEC O157:NM isolates to Caco-2 cells in the presence of anti-curli monoclonal antibody. Furthermore, the absence of curli expression in a *csgBA* deletion strain reduced the binding capacity of this strain to a level comparable to that of the NSF VTEC O157:H7 isolates tested. These data indicate that, following culture on CFA agar at 37°C, curli expression was the principal factor responsible for the adherence of SF VTEC O157:NM isolates to Caco-2 cells.

Chapter 6

Investigation of curli expression by sorbitol-fermenting *Escherichia coli* O157:NM isolates

The observation that SF VTEC O157:NM isolates recovered from patients in Scotland expressed curli at 37ºC following culture on CFA agar prompted an investigation of the ability of a larger number of SF VTEC O157:NM isolates to express curli. This chapter describes the investigation of curli expression by European SF VTEC O157:NM isolates.

6.1 Analysis of curli expression by European SF VTEC O157:NM isolates

To determine whether curli expression at 37°C is a common attribute of SF VTEC O157:NM strains, a collection of 66 isolates, collected by Prof. H. Karch's group (Műnster University, Germany), were analysed for their potential to bind CR on CFA-CR indicator plates at 37°C. This initial analysis was performed by Ms T. Dransfield (University of Edinburgh) and Dr R. La Ragione (VLA Weybridge) and a total of 52/66 (79%) SF VTEC O157:NM isolates were assessed as positive for CR binding. My research confirmed that CR binding by these isolates was associated with surface curli expression by immunostaining with the anti-curli monoclonal antibody. Two CR-positive isolates (E02/879 and E06/486) and two CR-negative isolates (340/97 and 080/01) were analysed and while both CR-positive isolates exhibited fluorescent surface staining, the two CR-negative isolates did not (Figure 6.1). Thus, curli expression on CFA agar at 37°C is a common property of SF VTEC O157:NM strains.

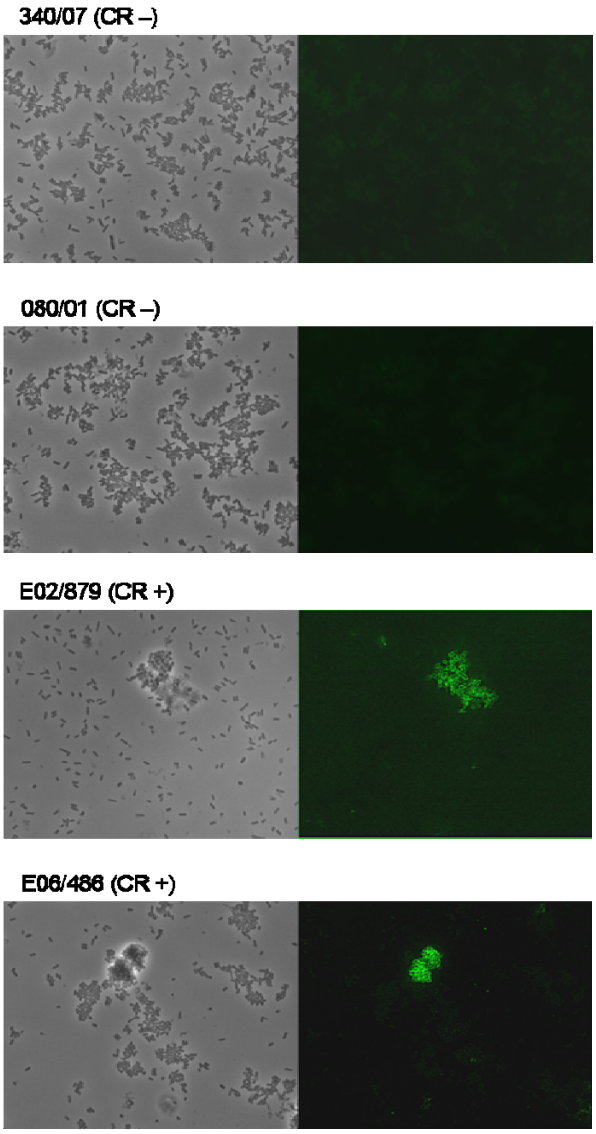


Figure 6.1. Detection of curli by immunofluorescence microscopy. Phase-contrast (left panel) and fluorescence (right panel) micrographs are shown for the indicated European SF VTEC O157:NM isolates stained for curli using an anti-curli monoclonal antibody following culture on CFA medium at 37°C. The potential of the isolate to bind CR on CFA-CR indicator plates at 37°C is indicated in parenthesis. Slides were examined using an x100 objective and images were captured with Leica software.

6.2 Contribution of curli expression to Caco-2 cell adherence

To further examine the association of curli expression with Caco-2 cell adherence, two CR-positive isolates and two CR-negative isolates were tested in Caco-2 adherence assays. Following culture on CFA agar at 37ºC, both CR-positive curli-expressing isolates (E02/879 and E06/486) adhered to Caco-2 cells at significantly higher levels ($P < 0.01$) than the CR-negative isolates that did not express curli (340/97 and 080/01) (Figure 6.2).

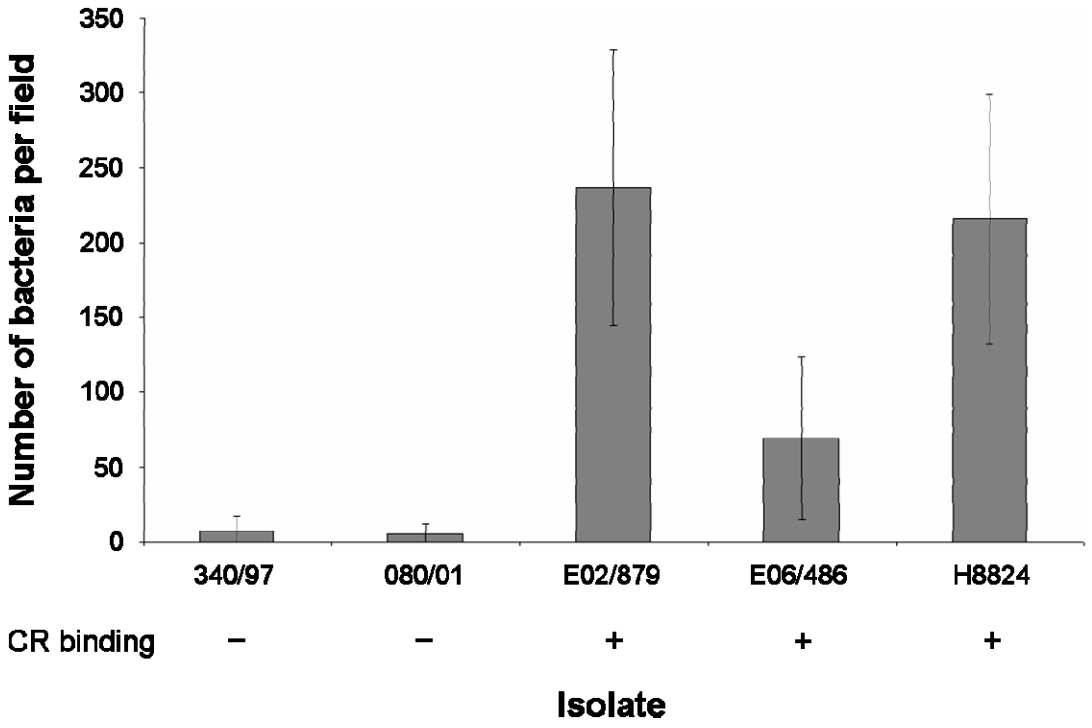


Figure 6.2. Adherence of selected European SF VTEC O157:NM isolates to Caco-2 cells. The indicated isolates were cultured on CFA agar at 37°C and adherence assays were performed at 37°C for 2 h. SF VTEC O157:NM isolate H8824 was included as a positive control. Microscopy was used to determine the number of bacteria per field for 30 fields. Adherence assays for each isolate were performed on at least three separate occasions. The bars represent the average number of bacteria per field and error bars define standard deviations. The potential of the isolate to bind CR on CFA-CR indicator plates at 37°C, which correlates with curli expression, is indicated below the graph.

6.3 Investigation of the differential curli expression in SF VTEC O157:NM isolates

The fact that there are differences in curli expression between SF VTEC O157:NM strains prompted an investigation to identify the source of this variation.

6.3.1 Nucleotide sequencing of curli operons

To establish if sequence variation in curli loci could account for the differences in curli expression between SF VTEC O157:NM isolates, the nucleotide sequences of the relevant regions (*csgBAC* and *csgDEFG* operons and the *csgB*-to-*csgD* intergenic region) were determined for CR-negative isolates 340/97 and 080/01 and CR-positive isolates E06/486 and H8824. GenBank accession numbers are detailed in Table 2.3. The sequences of the entire region from isolates 080/01, E06/486 and H8824 were identical, and isolate 340/97 only differed from them by a single nucleotide in the *csgD* gene. The putative CsgG, CsgF, CsgE, CsgD, CsgB, CsgA and CsgC proteins encoded by the four SF VTEC O157:NM isolates should be identical.

6.3.2 Measurement of *csgBAC* and *csgD* promoter activity in SF VTEC O157:NM isolates

Given that the curli expression differences are not accounted for by mutations or changes in the curli operons themselves, it is likely that the regulation acting on the *csgBAC* promoter must differ between these isolates. This could be on the *csgBAC* promoter via differences in CsgD activator levels or on the *csgBAC* promoter independent of the CsgD level (Barnhart & Chapman, 2006; Uhlich *et al.*, 2001). To investigate the expression of *csgBAC* and *csgD* in wild-type SF VTEC O157:NM isolates, promoter-GFP fusion constructs were transformed into different SF VTEC O157:NM backgrounds. Following the culture of transformed strains on CFA-CAM agar plates at 37°C, expression from the *csgBAC* promoter was found to be significantly higher ($P < 0.02$) in isolates E02/879, E06/486 and H8824 than in isolates 340/97 and 080/01 (Figure 6.3). These results correlate with CR binding data and curli expression. In contrast, while expression from the *csgD* promoter was significantly higher ($P < 0.001$) in one isolate (H8824), which could account for the

increase in *csgBAC* expression and curli production, it was equivalent in the four other isolates tested, only two of which express curli on CFA medium at 37°C (Figure 6.3). This indicates that curli expression in these isolates may be driven by regulators other than CsgD acting directly on the *csgBAC* promoter.

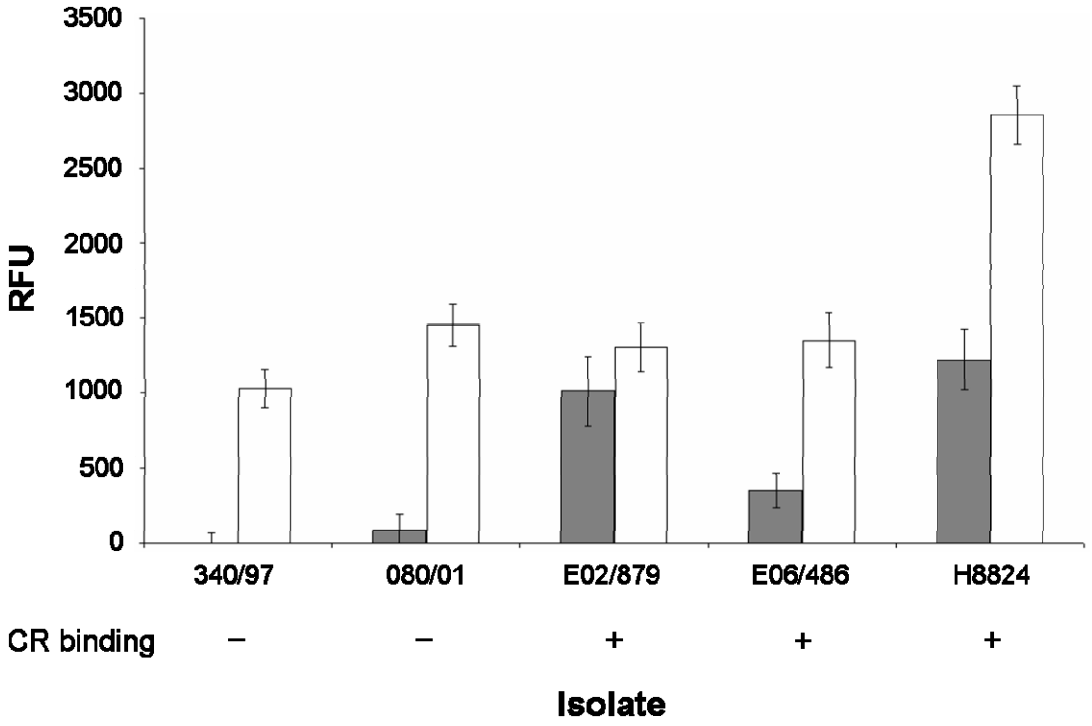


Figure 6.3. *csgBAC* and *csgD* promoter activity in selected European SF VTEC O157:NM isolates. The indicated isolates were transformed with plasmid-based promoter-GFP fusion constructs, and expression from the *csgBAC* promoter (shaded bars) and the *csgD* promoter (unshaded bars) was determined following the culture of transformed isolates on CFA-CAM agar at 37°C. Background fluorescence levels were determined for the promoter-less plasmid pAJR70 and subtracted. The data are expressed as relative fluorescence units (RFU). Error bars indicate standard errors of the means. The potential of the isolate to bind CR on CFA-CR indicator plates at 37°C, which correlates with curli expression, is indicated below the graph.

6.4 Discussion

SF VTEC O157:NM isolates recovered from clinical cases in Scotland were shown to express curli at 37°C following culture on CFA agar and this curli expression was found to be the main factor responsible for the increased adherence of SF VTEC O157:NM isolates to Caco-2 cells compared to NSF VTEC O157:H7 isolates. However, it is important to determine whether curli expression at 37°C is a common characteristic of SF VTEC O157:NM strains. This chapter described the investigation of curli expression by European SF VTEC O157:NM isolates.

Analysis, performed by Ms T. Dransfield and Dr R. La Ragione, of 66 SF VTEC O157:NM isolates recovered in Europe indicated that 79% could bind CR when cultured on CFA agar at 37°C. For the subset tested, this phenotype again correlated with curli expression, as determined by immunostaining with an anti-curli monoclonal antibody and *csgBAC* promoter fusion analysis. An interesting observation to come out of this analysis is that SF VTEC O157:NM isolates recovered most recently (from 2001 to present) were generally able to express curli under the conditions investigated whereas those recovered prior to 2001 generally were not. Therefore, curli expression at 37°C is a common attribute of recent SF VTEC O157:NM isolates following culture on CFA agar. Analysis of these isolates in Caco-2 adherence assays provided further evidence of the association of curli expression with Caco-2 cell adherence. Both CR-positive curli-expressing isolates adhered to Caco-2 cells at significantly higher levels than the CR-negative isolates that did not express curli.

The observation that many of the early SF VTEC O157:NM isolates did not express curli following culture on CFA agar at 37°C prompted an investigation of the differences in curli expression between SF VTEC O157:NM isolates. In *E. coli*, two divergently transcribed operons, *csgBAC* and *csgDEFG*, are required for curli formation (Hammar *et al.*, 1995). The *csgBAC* operon encodes: CsgA, the main subunit protein; CsgB, the nucleator protein; and CsgC, which has no reported role in curli formation (Barnhart & Chapman, 2006). The *csgDEFG* operon encodes four accessory proteins required for curli assembly, where CsgD is the positive

transcriptional regulator of the *csgBAC* operon (Barnhart & Chapman, 2006). The curli loci from four SF VTEC O157:NM isolates (two curli-expressing isolates and two that did not express curli under the conditions tested) were sequenced, and there were no changes detected that could account for the differences in curli expression. In particular, there were no sequence differences in the *csgB*-to-*csgD* intergenic region. This is significant as nucleotide changes in the *csgD* promoter region have been associated with variation in curli expression in *E. coli* O157:H7 (Uhlich *et al.*, 2001). Transcription from the *csgBAC* promoter requires expression of the CsgD positive regulator (Hammar *et al.*, 1995). In the present study, *csgBAC* expression was higher in isolates expressing curli. In one case, this increased level coincided with a higher level of *csgD* expression, but in the other isolates, levels of *csgD* expression were equivalent irrespective of curli expression phenotype. Therefore, it appears that curli expression in SF VTEC O157:NM isolates can be controlled either by increased *csgD* expression or by regulators acting directly on the *csgBAC* promoter together with CsgD. Curli expression is controlled by osmolarity and various stress responses, in particular those that affect membrane integrity (Barnhart & Chapman, 2006). Regulators controlled by these responses can act directly on the *csgBAC* promoter; for example, RcsB and CpxR, which are modified by phosphorylation (Barnhart & Chapman, 2006). It would be interesting to investigate differences in signaling pathways that control curli expression in SF VTEC O157:NM strains.

6.5 Section II discussion

The emergence of SF VTEC O157:NM strains in Europe is of particular concern since these pathogens appear to be associated with a higher incidence of HUS compared to the more common NSF VTEC O157:H7. The aim of this research was to investigate potential bacterial factors which could account for increased virulence of SF VTEC O157:NM strains. While no evidence of verocytotoxin or verocytotoxin expression differences between the two VTEC O157 groups were found, SF VTEC O157:NM isolates adhered at significantly higher levels to a human colonic cell line than NSF VTEC O157:H7 isolates. It was demonstrated that curli were the main factor responsible for this increased adherence observed *in vitro*. However, the ability of SF VTEC O157:NM bacteria to express functional type 1 fimbriae and Sfp fimbriae on their surface (this study; Musken *et al.*, 2008; Shaikh *et al.*, 2007) suggests that these adhesins are also likely to contribute to the adherence of these pathogens *in vivo*.

Although previous studies have shown that in *E. coli* curli are generally expressed optimally at temperatures below 30°C (Olsen *et al.*, 1989), this study demonstrated that SF VTEC O157:NM strains can express curli at 37°C. There is clear evidence that curli are an important virulence factor. For example, CR-binding variants of *E. coli* O157:H7 were more virulent in a mouse model (Uhlich *et al.*, 2002) and expression of curli at 37°C has been reported for a significant number of human *E. coli* sepsis isolates (Bian *et al.*, 2000). Curli expression may contribute to the virulence of the pathogen in several ways. Firstly, curli can contribute to increased colonisation through adherence to host cells (this study; Kikuchi *et al.*, 2005; Kim & Kim, 2004) and extracellular matrix proteins (Barnhart & Chapman, 2006; Olsen *et al.*, 1989) and through bacterial aggregation. Secondly, the interaction of curliated bacteria with certain host proteins may facilitate the spread of the bacteria through the host (Barnhart & Chapman, 2006) and as bacteria expressing curli can bind to human contact-phase proteins, including fibrinogen, the clotting cascade may be inhibited (Barnhart & Chapman, 2006). Thirdly, curli can activate the innate immune system and have been shown to be a pathogen-associated molecular pattern recognised by TLR2 (Tukel *et al.*, 2005). This is in agreement with an earlier study

which demonstrated that curli induce the proinflammatory cytokines TNF-α, IL-6 and IL-8 (Bian *et al.*, 2000). This final point may be critical in terms of toxin translocation during SF VTEC O157:NM infections as there is evidence that toxin circulates on the surface of neutrophils (te Loo *et al.*, 2000). Therefore, the more inflammation that is induced by bacteria in the gastrointestinal tract, the more neutrophils are recruited that could traffic toxin into the bloodstream via the lymph system.

It is noteworthy that curli expression at 37°C is characteristic of SF VTEC O157:NM isolates recovered most recently (from 2001 to present). Those isolates recovered prior to 2001 generally do not express curli under the conditions investigated. This is interesting since there were only two registered HUS outbreaks caused by SF VTEC O157:NM before 2001 in Germany (the first in 1988 and the other in 1996) whereas there were four HUS outbreaks caused by this pathogen during 2002 to 2006 (H. Karch, personal communication). It is possible that SF VTEC O157:NM have evolved the ability to express curli and this has provided strains with an increased virulence potential directly. Furthermore, perhaps the development of curli expression is a reflection of changes in the regulation and expression of other virulence genes by SF VTEC O157:NM and these also contribute to the pathogenicity of this organism. Changes in the regulation of virulence factors, including curli, may be a consequence of the acquisition or loss of DNA which carries effector proteins or regulators.

To support the role of curli in the disease process following infection with SF VTEC O157:NM, it would be interesting to analyse serum samples from infected patients to look for anti-curli antibodies.

The ability of SF VTEC O157:NM to express fimbrial adhesins and colonise the human intestine at higher levels could have serious implications for human health where the presence of greater bacterial numbers, and perhaps more persistent colonisation, in the intestine leads to increased toxin exposure and an increased likelihood of progression to HUS.

Conclusion

In attempting to understand differences in the pathogenic potential of bacterial strains, in a bid to minimise the risk of human infections, we are often in the situation of trying to develop an understanding of a complex set of interactions between the bacteria, their host and environment. In this situation, I have deliberately chosen to investigate the bacterial factors that are of importance to the emergence of two bacterial pathogens, although this work is informed by some host and environmental information. It remains a key requirement to place my work into the context of human health in Scotland.

This research illuminated key bacterial differences which may potentially contribute directly to the increased pathogenicity of a strain, or which simply serve as indicators of other differences. However, even when focusing on solely bacterial factors, we find ourselves in a complex situation since a single *E. coli* serotype is a complex mixture of strains with different combinations of virulence-associated determinants that have evolved together to make a successful pathogen. Developing an understanding of the synergism among different factors is essential.

However, in spite of this complexity, curli expression has been identified as a key virulence factor for SF VTEC O157:NM. This research demonstrated that SF VTEC O157:NM can express curli at 37°C following culture on CFA agar whereas NSF VTEC O157:H7 do not. Since Congo red (CR) binding is a well established method to demonstrate surface curli expression and CFA-CR indicator plates were able to distinguish the majority of SF VTEC O157:NM isolates from NSF VTEC O157:H7 at 37°C, it is possible that CFA-CR indicator plates could be used as a basis for the detection and identification of SF VTEC O157:NM in a diagnostic laboratory and this merits further investigation.

This study demonstrated that curli are expressed by the vast majority of recently isolated SF VTEC O157:NM strains. Therefore, it is recommended that an examination of European strain repositories of SF VTEC O157:NM isolates should

be conducted to identify if the evolution of isolates capable of expressing curli at 37°C is a relatively recent event.

Since curli is a proven virulence factor that may promote increased pathogenicity in a strain in a number of ways (see section 6.5 for details), it would be useful to develop a good animal model that could be used to analyse strains that do and do not express curli at 37°C to get a better understanding of the role of curli in pathogenesis.

In the study of *E. coli* O26 strains it is apparent that the genetic backbone of strains is relatively similar. However, the work did reveal fundamental attributes that may be considered important for strains to be more likely to cause serious human infection. These attributes include the carriage of the gene encoding verocytotoxin 2, allele 1 for the region upstream of *LEE1* and also possession of *tccP2*. Thus, I consider that it would be beneficial for there to be greater screening of *E. coli* O26 isolates from cattle, and other potential reservoirs, for strains carrying the above mentioned virulence determinants, and that the Reference Laboratory should include these characterisations in its routine screening of HUS cases.

Finally, because of the complexity of bacterial pathogenesis it is not possible to be certain about the threat to human health of a particular pathogen. However, I have shown that *E. coli* strains which may represent a threat are present in Scotland and, since bacteria are continually evolving, there will no doubt be the emergence of new pathogenic clones in the future. Continued surveillance and further research will be needed to monitor this threat.

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Appendix 1: Preparation of media and reagents

All chemicals used were from Sigma-Aldrich unless otherwise stated.

CDMT broth

A concentrated stock of each component was prepared and autoclaved. The working medium contained 13 mM K_2HPO_4, 6 mM KH_2PO_4, 8 mM $(NH_4)_2SO_4$, 2 mM sodium citrate, 0.4 mM $MgSO_4$ (Fisher), 0.2% (w/v) casamino acids (Oxoid), 0.2% (w/v) glucose, 5 µM $CaCl_2$ and 0.01% (w/v) tryptone (BD Biosciences).

CFA agar

Medium consisting of 1% (w/v) casamino acids (Oxoid), 0.15% (w/v) yeast extract (Oxoid) and 1.5% (w/v) agar (Melford) was autoclaved and cooled to 55°C before the following sterile components were added: 0.005% (w/v) $MgSO_4$ (Fisher) and 0.0005% (w/v) $MnCl_2$.

CFA broth

As for CFA agar without the agar.

Crystal violet solution

Crystal violet stock: 0.5% (w/v) crystal violet prepared in dH_2O was filtered through Whatman filter paper and phenol [0.25% (w/v), BDH] added.

Crystal violet solution: 0.13% crystal violet, 0.065% phenol, 5% (v/v) ethanol, 2% (v/v) formaldehyde in PBS. [To prepare 200 ml: 52 ml crystal violet stock, 10 ml ethanol, 10.8 ml 37% formaldehyde in 127.2 ml PBS].

ECL solution

ECL solution was made by mixing equal volumes of ECL reagent 1 and ECL reagent 2.

ECL reagent 1: 100 mM Tris-HCl (pH 8.5), 2.5 mM Luminol (Fluka), 0.4 mM *p*-coumaric acid.

ECL reagent 2: 100 mM Tris-HCl (pH 8.5), 0.02% (v/v) hydrogen peroxide.

Soft agar

1% trypticase peptone (BD Biosciences), 0.5% NaCl, 0.3% agar (Melford).

TFB I

The final 1 L solution contained 30 mM potassium acetate ($KC_2H_3O_2$), 10 mM $CaCl_2.2H_2O$, 100 mM KCl, 15% (v/v) glycerol and 50 mM $MnCl_2$. The potassium acetate, $CaCl_2.2H_2O$, KCl and glycerol were prepared in a volume of 900 ml and autoclaved before 100 ml sterile $MnCl_2$ was added.

TFB II

75 mM $CaCl_2$, 10 mM KCl, 15% (v/v) glycerol and 10 mM Na-MOPS (pH 7.0).

Appendix 2: Genotypic characterisation of *E. coli* O26 isolates.

Isolate[a]	Clinical Info[b]	MLST				Detection of virulence genes by PCR[e]				
		ST[c]	ST cplx[c]	*espA*[d]	upstream of *LEE1*[d]	*vtx*[f]	LEE[g]	*hlyA*	*tccP*	*tccP2*[h]
ZAP1077_B1		29	29	1	3	–	+	–	–	+ (1050bp)
ZAP1078_B2		21	29	1	1	1, 2	+	+	–	–
ZAP1079_B3		21	29	1	2	1	+	+	–	–
ZAP1080_B4		21	29	1	2	1	+	+	–	+ (750bp)
ZAP1081_B5		21	29	1	2	1	+	+	–	+ (750bp)
ZAP1082_B6		29	29	1	1	–	+	+	–	+ (1050bp)
ZAP1083_B7		21	29	1	2	1	+	+	–	–
ZAP1084_B8		21	29	1	2	1	+	+	–	–
ZAP1085_B9		21	29	1	2	1	+	+	–	–
ZAP1086_B10		21	29	1	2	1, 2	+	+	–	–
ZAP1087_B11		986	10	ND	ND	1	–	–	–	–
ZAP1088_B12		21	29	1	2	1	+	+	–	–
ZAP1089_B13		29	29	1	4	–	+	+	–	+ (750bp)
ZAP1090_B14		29	29	1	1	–	+	–	–	+ (1050bp)
ZAP1091_B15		21	29	1	2	1	+	+	–	+ (750bp)
ZAP1092_B16		29	29	1	4	–	+	+	–	+ (900bp)
ZAP1093_B17		21	29	1	2	1	+	+	–	–
ZAP1094_B18		21	29	1	2	1, 2	+	+	–	–
ZAP1095_B19		21	29	1	2	1	+	+	–	+ (750bp)
ZAP1096_B20		29	29	1	1	–	+	+	–	+ (1050bp)
ZAP1097_B21		21	29	1	2	1	+	+	–	–
ZAP1098_B22		29	29	1	4	–	+	+	–	+ (750bp)
ZAP1099_B23		29	29	1	1	–	+	–	–	+ (1050bp)
ZAP1100_B24		21	29	1	2	1, 2	+	+	–	–

Appendix 2 *continued*

Isolate[a]	Clinical Info[b]	MLST				Detection of virulence genes by PCR[e]				
		ST[c]	ST cplx[c]	*espA*[d]	upstream of *LEE1*[d]	*vtx*[f]	LEE[g]	*hlyA*	*tccP*	*tccP2*[h]
ZAP1101_B25		21	29	1	2	1	+	+	–	+ (750bp)
ZAP1102_B26		29	29	1	1	–	+	–	–	+ (1050bp)
ZAP1103_B27		21	29	1	2	1	+	+	–	+ (750bp)
ZAP1104_B28		10	10	ND	ND	–	–	–	–	–
ZAP1105_B29		21	29	1	1	1, 2	+	+	–	+ (750bp)
ZAP1106_B30		21	29	1	2	1, 2	+	+	–	–
ZAP1107_B31		21	29	1	2	1	+	+	–	–
ZAP1108_B32		21	29	1	2	1	+	+	–	+ (750bp)
ZAP1109_B33		21	29	1	2	1, 2	+	+	–	–
ZAP1111_S2	No info	574	29	1	2	1	+	+	–	–
ZAP1112_S3	D	29	29	1	4	–	+	+	–	+ (900bp)
ZAP1114_S6	D	21	29	1	2	1	+	+	–	–
ZAP1115_S7	D	21	29	1	2	1	+	+	–	–
ZAP1116_S8	BD	29	29	1	1	–	+	–	–	+ (1050bp)
ZAP1117_S9	D	10	10	ND	ND	–	–	–	–	–
ZAP1118_S10	D	29	29	2	5	–	+	+	–	+ (950bp)
ZAP1119_S11	D	575	29	1	1	–	+	+	–	+ (1250bp)
ZAP1120_S12	D	29	29	1	6	–	+	–	–	+ (1400bp)
ZAP1152_S35	D	21	29	1	2	1, 2	+	+	–	–
ZAP1153_S36	BD	21	29	1	2	1, 2	+	+	–	–
ZAP1121_G13	D	29	29	1	1	–	+	–	–	+ (900bp)
ZAP1122_G14	HUS	21	29	1	2	1, 2	+	+	–	+ (750bp)
ZAP1124_G16	HUS	21	29	1	2	1	+	–	–	+ (750bp)
ZAP1125_G17	D	29	29	1	1	–	+	+	–	+ (900bp, 750bp)

Appendix 2 *continued*

Isolate[a]	Clinical info[b]	MLST				Detection of virulence genes by PCR[e]				
		ST[c]	ST cplx[c]	*espA*[d]	upstream of *LEE1*[d]	*vtx*[f]	LEE[g]	*hlyA*	*tccP*	*tccP2*[h]
ZAP1126_G18	HUS	21	29	1	1	1, 2	+	–	–	+ (750bp)
ZAP1127_G19	D	21	29	1	2	1	+	+	–	+ (750bp)
ZAP1128_G20	HUS	29	29	1	1	2	+	+	–	+ (850bp)
ZAP1129_G21	HUS	21	29	1	2	1, 2	+	–	–	+ (750bp)
ZAP1130_G22	HUS	21	29	1	1	1, 2	+	+	–	+ (750bp)
ZAP1131_G23	D	21	29	1	1	1	+	–	–	+ (750bp)
ZAP1132_G24	HUS	21	29	1	2	2	+	–	–	–
ZAP1133_G25	D	21	29	1	1	–	+	–	–	+ (900bp)
ZAP1134_G26	HUS	29	29	1	1	2	+	+	–	+ (900bp)
ZAP1135_G27	D	29	29	1	1	–	+	+	–	+ (900bp)
ZAP1146_I29	HUS	21	29	1	1	1	+	+	–	–
ZAP1147_I30	HC	21	29	1	1	2	+	+	–	+ (900bp)
ZAP1149_I32	HUS	21	29	1	1	2	+	+	–	+ (900bp)
ZAP1150_I33	HUS	21	29	1	1	2	+	+	–	+ (900bp)
ZAP1151_I34	HUS	21	29	1	1	1	+	+	–	+ (750bp)

[a] The suffix indicates the origin of the isolate: B, bovine; S, human (Scotland); G, human (Germany); I, human (Italy).

[b] Information provided by the supplier of the isolate. BD, bloody diarrhoea; D, diarrhoea; HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome.

[c] The sequence type (ST) and ST complex were assigned in accordance with the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli) following sequencing of housekeeping genes.

[d] The allele numbers for *espA* and the region upstream of *LEE1* were assigned in the order in which they were discovered; ND, not determined.

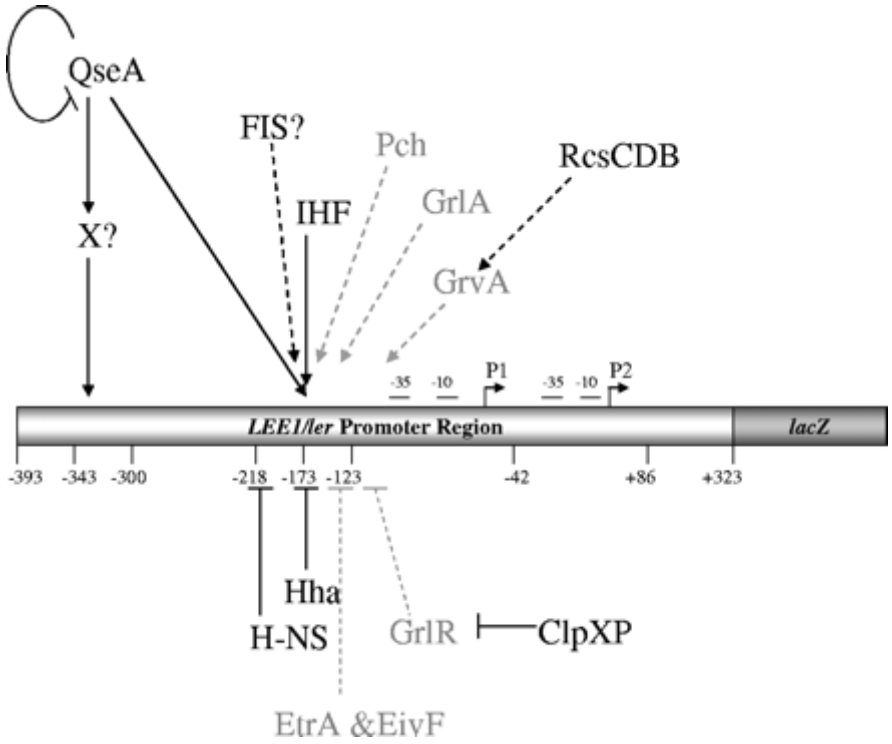
[e] +, gene present; –, gene absent.

[f] *vtx* types: 1, vtx_1; 2, vtx_2; 1, 2, vtx_1 and vtx_2; –, *vtx* negative.

[g] The possession of the LEE was determined by PCR screening for *eae* (intimin) and *sepL* (SepL).

[h] PCR fragment size(s) are indicated in parentheses.

Appendix 3



Appendix 3. Schematic diagram detailing factors involved in the regulation of the LEE in *E. coli* O157:H7. The numbering of the bases (-393 to +323) is in relation to the P2 *LEE1* promoter transcriptional start site. Image reproduced from Sharp and Sperandio (2007).

Appendix 4